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Perfluorododecanoic Acid-Induced Steroidogenic Inhibition is Associated with Steroidogenic Acute Regulatory Protein and Reactive Oxygen Species in cAMP-Stimulated Leydig Cells

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Perfluorododecanoic acid (PFDoA) can be detected in environmental matrices and human serum and has been shown to inhibit testicular steroidogenesis in rats. However, the mechanisms that are responsible for the toxic effects of PFDoA remain unknown. The aims of this study were to investigate the mechanism of steroidogenesis inhibition by PFDoA and to identify the molecular target of PFDoA in Leydig cells. The effects of PFDoA on steroid synthesis in Leydig cells were assessed by radioimmunoassay. The expression of key genes and proteins in steroid biosynthesis was determined by real-time PCR and Western blot analysis. Reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) levels were determined using bioluminescence assays. PFDoA inhibited adenosine 3′,5′-cyclophosphate (cAMP)-stimulated steroidogenesis in mouse Leydig tumor cells (mLTC-1) and primary rat Leydig cells in a dose-dependent manner. However, PFDoA (1–100μM) did not exhibit effects on cell viability and cellular ATP levels in mLTC-1 cells. PFDoA inhibited steroidogenic acute regulatory protein (StAR) promoter activity and StAR expression at the messenger RNA (mRNA) and protein levels but did not affect mRNA levels of peripheral-type benzodiazepine receptor, cholesterol side-chain cleavage enzyme, or 3β-hydroxysteroid dehydrogenase in cAMP-stimulated mLTC-1 cells. PFDoA treatment also resulted in increased levels of mitochondrial ROS and H_2O_2. After excessive ROS and H_2O_2 were eliminated in PFDoA-treated mLTC-1 cells by MnTMPyP (a superoxide dismutase analog), progesterone production was partially restored and StAR mRNA and protein levels were partially recovered. These data show that PFDoA inhibits steroidogenesis in cAMP-stimulated Leydig cells by reducing the expression of StAR through a model of action involving oxidative stress.

Key Words: **PFDoA; steroidogenesis; StAR; ROS.**

Perfluoroalkyl acids (PFAAs) are perfluorinated chemicals that have been produced and used as cosmetics, lubricants, fire retardants, insecticides, and surfactants (Lau *et al.*, 2007; Renner, 2001). PFAAs comprise a series of compounds with different carbon chain lengths, including perfluorododecanoic acid (PFDoA, C12), perfluorodecanoic acid (PFDA, C10), perfluorooctanoic acid (PFOA, C8), and perfluorooctane sulfonic acid (PFOS, C8). PFAAs persist in the environment and can be detected in wildlife and in humans (Lau *et al.*, 2007). For example, PFDoA was detected in human breast milk in Massachusetts and in the livers of harbor porpoises in the Black Sea, with the highest detected concentrations reaching 9.74 pg/ml and 9.5 ng/g (wet weight), respectively (Tao *et al.*, 2008; Van de Vijver *et al.*, 2007). The highest concentration detected in human sera was 0.022 ng/ml in samples from Sri Lanka (Guruge *et al.*, 2005). The potential health risk of PFAAs to the human population has raised concern among environmental agencies. Indeed, PFAA exposure in laboratory animals can lead to toxic effects, including body weight reduction, liver hypertrophy, lung toxicity, tumorigenicity, and developmental toxicity (Lau *et al.*, 2004; Kudo and Kawashima, 2003a).

In rodent models, PFAAs have been shown to cause testicular toxicity by disrupting steroidogenesis. PFOA decreases testosterone levels in serum and testicular interstitial fluid and concomitantly raises increased serum estradiol levels in male rats (Biegel *et al.*, 1995). Chronic exposure of PFOA (300 ppm) in the diets of male rats induced Leydig cell adenomas (Biegel *et al.*, 2001). The testes have also been suggested as being a potential target for PFDA (Olson and Andersen, 1983). PFDA decreases plasma testosterone and 5α-dihydrotestosterone concentrations in a dose-dependent fashion in male rats, with a 50% median effective dose (ED_{50}) of approximately 30 mg/kg (Bookstaff *et al.*, 1990). PFDA decreases human chorionic gonadotropin (hCG)-stimulated progesterone synthesis in MA-10 cells in a dose- and time-dependent manner (Boujrad *et al.*, 2000). In our previous studies, we found that serum testosterone levels of male rats decreased after 14 days of PFDoA exposure at doses of 5 or 10 mg/kg/d (Shi *et al.*, 2007). Moreover, 110-day PFDoA exposure at 0.2 or 0.5 mg/kg/d also caused significantly decreased testosterone levels and changed the expression of testicular steroidogenic genes in rats (Shi *et al.*, 2009). These

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results clearly show that PFAAs affect the production of sex hormones, especially testosterone synthesis in male rats. However, the molecular mechanism by which PFAAs disrupt testosterone production in male rats remains unknown.

Relative to PFOA and PFDA, PFDoA may exhibit unique toxic effects since PFAAs with longer carbon chains generally persist in the body longer than shorter chain PFAAs (Kudo and Kawashima, 2003b; Ohmori *et al.*, 2003). Relative to PFOA, PFDoA has been shown to accumulate more in wildlife species (Senthilkumar *et al.*, 2007). However, little is known about the full-scale reproductive toxicity and related mechanisms of PFDoA in male animals. Although acute and chronic studies suggest that testosterone inhibition by PFDoA is associated with changes in the expression of genes responsible for cholesterol transport and steroidogenesis in the testes (Shi *et al.*, 2007; 2009), the precise mechanism of testosterone inhibition by PFDoA and the molecular target of PFDoA activity remain unknown.

Mouse Leydig tumor cells (mLTC-1) have been used extensively to study the regulation of steroidogenesis because they remain responsive to luteinizing hormone (LH)/hCG and cAMP and because they express some of the key genes of steroidogenesis, such as steroidogenic acute regulatory protein (StAR), P450 side-chain cleavage enzyme (P450scc), and 3β-hydroxysteroid dehydrogenase (3β-HSD) (Manna *et al.*, 2006). mLTC-1 cells primarily produce progesterone and very low levels testosterone (Panesar *et al.*, 2003).

The purpose of this study was to use mLTC-1 cells as a model to investigate the effects of PFDoA on steroid production and to identify possible underlying mechanisms for these effects. We observed that PFDoA inhibited steroid synthesis in a dose-dependent fashion in Leydig cells. PFDoA also suppressed the expression of StAR at the messenger RNA (mRNA) and protein levels and increased levels of mitochondrial reactive oxygen species (ROS) in cAMP-stimulated mLTC-1 cells. The elimination of excessive ROS resulted in a partial recovery of StAR expression and progesterone synthesis in PFDoA-treated cells. StAR and ROS play important roles in PFDoA-induced steroid inhibition.

MATERIALS AND METHODS

Reagents. PFDoA (CAS No.307-55-1, 95% purity), 22R-hydroxycholesterol (22R-HC), 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and trypan blue were purchased from Sigma-Aldrich (St Louis, MO). RPMI 1640 medium was purchased from HyClone (Logan, UT). Standard fetal bovine serum was purchased from Sijiqing (Hangzhou, China). PFDoA was first dissolved in alcohol and then adjusted to a concentration of 1mM with serum-free RPMI 1640 medium to generate a stock solution. In formal experiments, the final concentration of alcohol was 0.01%. All other chemicals used were of analytical grade.

Leydig cell culture and treatments. mLTC-1 cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium containing 2.05mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 g/ml streptomycin at 5% CO_2 and 37°C. To assess the dose effects of PFDoA, mLTC-1 cells were grown in culture to approximately 70% confluency and fresh media containing increasing concentrations of PFDoA (1–500μM) was added for 24 h. To assess the time course of PFDoA action, the cells were plated at a low density for 24 h and then treated with fresh media containing the indicated PFDoA concentrations (1–100μM) for 24, 48, or 72 h. In all cases, an alcohol concentration of 0.01% was not cytotoxic on its own. At the end of the incubation, the media was removed and the cells were then washed three times with PBS and stimulated for 3 h with dbcAMP (1mM) in serum-free medium. cAMP at a concentration of 1mM was enough to stimulate steroid synthesis in these cells. At the end of the 3-h incubation period, the cell media was collected for progesterone determination and the cells were either dissolved in lysis buffer containing the protease inhibitor phenylmethylsulfonyl fluoride for protein determination or prepared for other biochemical assays.

Isolation of rat Leydig cells was performed as previously described (Manna *et al.*, 2001; Mondillo *et al.*, 2009). Briefly, freshly harvested testes obtained from six adult Sprague-Dawley (SD) rats (average body weight: 300 g) were decapsulated and dispersed by collagenase dissociation (0.5 mg/ml). Leydig cells were further purified by discontinuous Percoll (GE Healthcare Bio-Sciences, Uppsala, Sweden) gradient (density range 1.01–1.126 g/l) centrifugation at 800 × g for 30 min, according to a previous description (Mondillo *et al.*, 2009). The cell types at a specific gravity of 1.07 of Percoll were collected. The preparations contained 70–80% Leydig cells, as determined by histochemical staining for 3β-HSD (Klinefelter *et al.*, 1987). Leydig cells were cultured for 24 h with PFDoA (1–500μM) under the same conditions as described above for mLTC-1 cells. At the end of the incubation, the cells were stimulated for 3 h with 1mM cAMP in serum-free medium. Media was collected for testosterone measurements.

Detection of progesterone and testosterone. The concentrations of progesterone and testosterone were determined by radioimmunoassay using commercial kits (Beijing North Institute of Biological Technology, Beijing, China). Cellular protein levels were quantified using the Bradford Kit (Applygen Technologies Inc., Beijing, China). Progesterone or testosterone concentrations for each sample were normalized to protein concentrations (nanogram per milligram protein) or cell numbers (ng/5 × 10^5 cells), respectively.

Analysis of cell viability and measurement of cellular ATP. Cell viability was analyzed by the trypan blue exclusion method. After the cells were treated as described above, they were washed with PBS, trypsinized at room temperature, and then resuspended in PBS. After an incubation of 15 min with 0.4% trypan blue stain, viable cells (unstained) and nonviable cells (stained) were counted using a standard hemocytometer by light microscopy. The percent cell viability was calculated by the formula [% cell viability = total viable cells/total cells × 100]. Cell viability was also evaluated by the MTT proliferation assay (Kleszczynski *et al.*, 2007). The results are presented as the percentage of the control values obtained using untreated cells. Cellular ATP was assayed using the ATP bioluminescence assay kit (Genmed Scientific Inc., Shanghai, China), according to the manufacturer's instructions. ATP concentrations were normalized to protein concentrations for each sample and expressed as micromolar per milligram protein.

Real-time PCR. Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Reverse transcription was performed using an oligo-$(dT)_{15}$ primer, deoxynucleotide triphosphate (deoxy-NTP), and the moloney murine leukemia virus reverse transcriptase, which were purchased from Promega (Madison, WI) in accordance with the manufacturer's recommendations. All real-time PCR reactions were carried out on a Stratagene Mx3000P (Stratagene, La Jolla, CA) using SYBR Green PCR Master Mix reagent kits (Takara, Dalian, China) according to the manufacturer's instructions for quantification of gene expression. Mouse-specific primers and reaction conditions for the genes of interest are listed in Supplementary table 1. The housekeeping gene β-actin was used as an internal control. The relative expression ratio of a target gene was calculated using the C_t value as described previously (Barlow *et al.*, 2003).

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Western blotting. Total cellular protein was obtained and quantified as described above. Proteins (20 μg/lane) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane (Amersham Hybond, Uppsala, Sweden). The blots were probed overnight at 4°C via exposure to a 1:1000 dilution of 30-kDa rabbit anti-rat polyclonal StAR antibodies (Abcam Inc., Cambridge, MA) and P450scc antibodies (Millipore Corp., Bedford, MA) or a 1:500 dilution of a mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were further incubated for 2 h at room temperature in the presence of horseradish peroxidase–conjugated anti-rabbit or anti-mouse antibodies (1:5000 dilution, Santa Cruz Biotechnology). Immune complexes were detected with an ECL Western Blot Detection Kit (Tiangen, Beijing, China) and visualized using x-ray film (Kodak, Rochester, NY). The intensities of the scanned bands were obtained by a Tanon Imager program (Shanghai Tanon Science and Technology, Shanghai, China) and were normalized to the β-actin signal.

StAR promoter activity analysis. The 5′-flanking-1224/-1-bp region of the promoter is sufficient for full cAMP responsiveness (Manna *et al.*, 2002; Wooton-Kee and Clark, 2000). The complete promoter was synthesized using a PCR-based cloning strategy and subcloned into the pGL3 basic vector (Promega), which contains the firefly luciferase gene as a reporter. The sense primer 5′-CTTGGTACCGTCTTGTCCTGCCTTGGGACAGTAA-3′ and the antisense primer 5′-CCCAAGCTTGGCGCAGATCAAGTGCGCTGCCTTA-3′ contain Kpn I and Hind III sites (underlined). Transfection was performed using Lipofectamine 2000 (Invitrogen) and 1 μg of the reporter gene in serum-free media. The transfection efficiency was normalized by cotransfecting 50 ng of pRL-SV40 vector (Promega). Twenty-four hours after transfection, the mLTC-1 cells were treated with or without 100μM PFDoA for 24 h in serum media and then were stimulated by 1mM cAMP or 1mM cAMP plus 250μM H_2O_2 for 3 h. Luciferase activity in the cell lysates was determined by the Dual-Luciferase Reporter Assay System (Promega) on a FLUOstar OPTIMA instrument (Offenburg, Germany) according to the manufacturer's recommendations.

Measurement of mitochondrial ROS and H_2O_2. Mitochondrial ROS levels in mLTC-1 cells were evaluated using an ROS assay kit according to the manufacturer's recommendations (Genmed Scientific Inc.). Mitochondria were isolated as described previously (Comelli *et al.*, 2007). 6-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) was used as the molecular probe for ROS determination by the FLUOstar OPTIMA instrument. ROS levels were normalized to total mitochondrial protein content for each sample and expressed as absolute fluorescence units per microgram protein.

Mitochondrial H_2O_2 levels were measured using the horseradish peroxidase–linked Amplex Red fluorescence assay as described previously (Komary *et al.*, 2008) with a minor modification. Briefly, 10μM Amplex Red (Invitrogen) and horseradish peroxidase type II (1 U/ml) were added to the medium, and mitochondria (50 μg) were then applied. H_2O_2 was produced by the addition of glutamate and malate (10mM each) or succinate (10mM). Fluorescence readings for triplicate samples were obtained at Ex/Em = 530/580 nm. The concentration of H_2O_2 was calculated using a standard curve and normalized to mitochondrial protein. Protein concentration was determined using a Bradford Kit as described above.

Statistical analyses. All data were analyzed using SPSS statistical software (SPSS, Inc., Chicago, IL). All results are presented as averages ± SEM. Statistical differences between control and treatment groups were evaluated by Dunnett's post hoc two-sided *t*-test. Differences were considered significant at $p < 0.05$.

RESULTS

Effect of PFDoA on Steroid Production in Leydig Cells

Steroid production in Leydig cells is primarily under the control of LH, which acts via its intracellular second messenger cAMP to acutely regulate progesterone or testosterone production at the level of cholesterol transport into the mitochondria. Therefore, in the present study, mLTC-1 cells were treated with increasing concentrations of PFDoA (1–500μM) for 24 h and then 1mM cAMP for 3 h in order to stimulate the Leydig cells *in vitro*. As shown in Figure 1A, 1mM cAMP treatment resulted in an approximately 250-fold increase in progesterone synthesis in mLTC-1 cells (control vs. cAMP: 4.2 ± 0.8 vs. 1015.6 ± 50.0 ng/mg protein). However, PFDoA inhibited cAMP-induced progesterone synthesis in a dose-dependent manner. Treatment of mLTC-1 cells with 10, 50, 100, and 500μM PFDoA caused significant decreases in cAMP-induced progesterone production relative to treatment with cAMP alone (36, 45, 53, and 57%, respectively, corresponding to 655.4 ± 38.6, 559.9 ± 41.4, 479.9 ± 28.2, and 438.5 ± 20.2 ng/mg protein [$p < 0.01$, $n = 6$]). However, when mLTC-1 cells were treated with PFDoA for 24 h, 48 h, or 72 h, no obvious time effect was observed between cAMP- and cAMP/PFDoA-treated cells (Fig. 1B). Considering these results, 24 h was selected as the PFDoA treatment time for exploring the mechanism of PFDoA action. We then examined the reversibility of the inhibitory activity of PFDoA on progesterone production. Following a 24-h treatment with 100μM PFDoA, the mLTC-1 cells were washed and further cultured in PFDoA-free medium for 24 h. At the end of the culture period, the cells were stimulated with 1mM cAMP for 3 h. The level of progesterone production in mLTC-1 cells treated for 24 h with PFDoA was recovered, reaching the level of cells treated with cAMP alone (data not shown). These results indicate that PFDoA did not exert a permanent inhibitory effect on steroidogenesis in mLTC-1 cells.

To determine whether the inhibitory effect of PFDoA on mLTC-1 cells is similar to that seen in primary adult rat Leydig cells, rat Leydig cells were isolated and cultured for 24 h in medium containing increasing concentrations of PFDoA (1–500μM). The results revealed that PFDoA inhibited cAMP-stimulated testosterone production in a dose-dependent manner (Fig. 1C). Moreover, this inhibitory effect was significant when the PFDoA concentration was equal to or greater than 10μM ($p < 0.01$, $n = 6$). These results were similar to the response of mLTC-1 cells to PFDoA, validating the use of mLTC-1 cells for elucidation of the inhibitory mechanism of PFDoA on Leydig cell steroidogenesis.

Effects of PFDoA on mLTC-1 Cell Viability and ATP Levels

As shown by trypan blue exclusion analysis, 1–100μM PFDoA did not affect the viability of mLTC-1 cells (Fig. 2A). This result was further confirmed by MTT analysis (Fig. 2B). In addition, 1mM cAMP or cAMP and PFDoA (1–100μM) did not exhibit any observable effects on ATP levels in mLTC-1 cells (Fig. 2C).

Effects of PFDoA on Steroidogenic Gene Expression in mLTC-1 Cells

In order to explore the molecular mechanism of PFDoA activity, we evaluated the expression of genes related to

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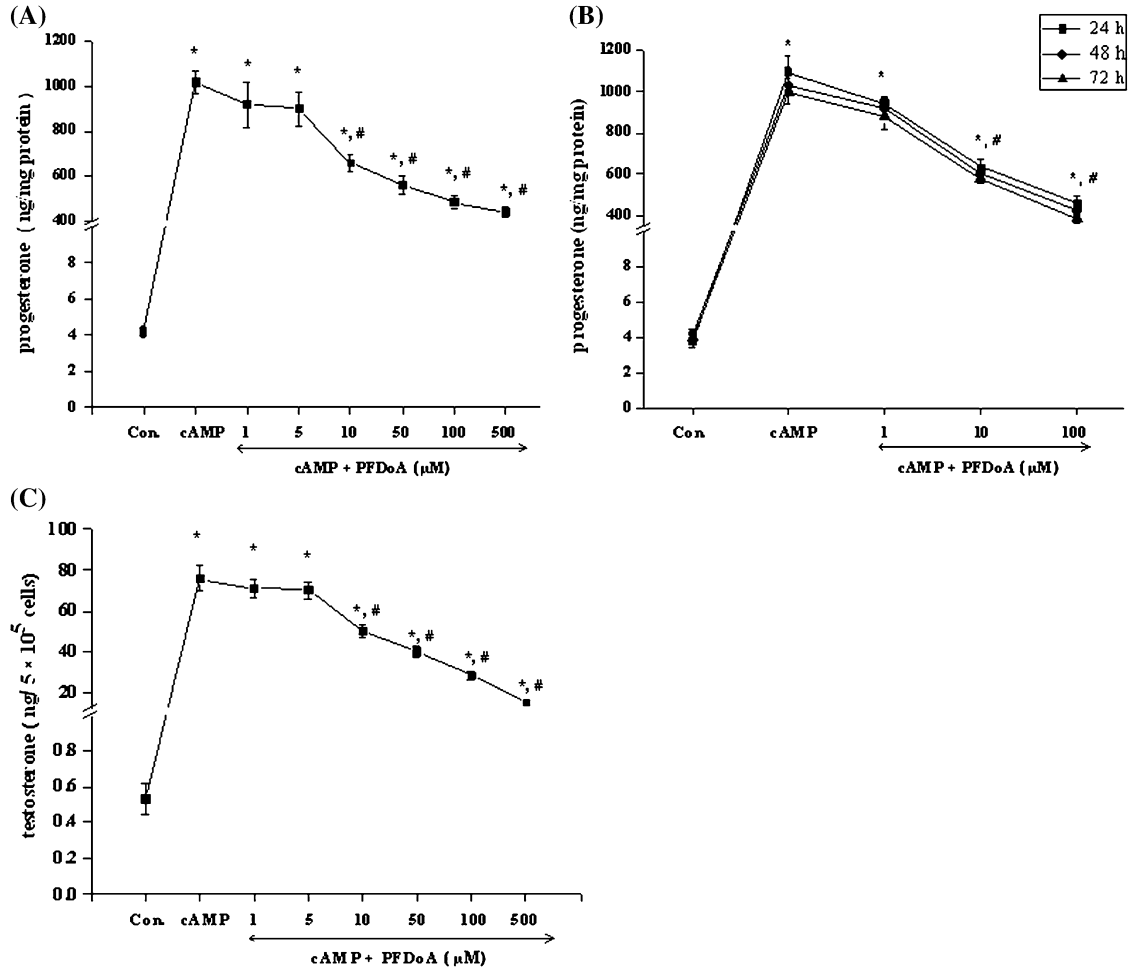

FIG. 1. Dose- and time-dependent effects of PFDoA on cAMP-stimulated steroid production in Leydig cells. Leydig cells were treated with PFDoA (1–500μM) for 24, 48, or 72 h, followed by stimulation with 1mM cAMP for 3 h. Values are presented as means ± SEM ($n = 6$) from three independent experiments. (A) Dose-dependent effect in mLTC-1 cells treated with PFDoA (1–500μM) for 24 h; (B) time-dependent effect in mLTC-1 cells; and (C) dose-dependent effect in primary rat Leydig cells; *significant difference from untreated controls ($p < 0.01$) and #significant difference from the cAMP group ($p < 0.01$).

steroidogenesis in mLTC-1 cells (Fig. 3). Relative to control cells, cAMP caused a 30-fold increase in StAR mRNA levels ($p < 0.01$, $n = 3$) but did not affect the mRNA levels of peripheral-type benzodiazepine receptor (PBR), P450scc, or 3β-HSD. Relative to cells treated with cAMP alone, 1–100μM PFDoA did not markedly affect the mRNA levels of PBR, P450scc, or 3β-HSD in mLTC-1 cells. However, 10 and 100μM PFDoA decreased StAR mRNA levels to 70 and 36%, respectively, of those in cAMP-treated cells ($p < 0.05$, $n = 3$). Relative to cAMP-treated cells, StAR protein abundance was significantly decreased by 42 and 59% in response to treatment with 10 and 100μM PFDoA, respectively ($p < 0.01$, $n = 3$; Fig. 4). StAR protein was virtually undetectable in control cells, but cAMP stimulated the production of StAR protein relative to that of controls (Fig. 4A). P450scc protein levels were not significantly affected by PFDoA. As StAR is responsible for the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane in Leydig cells and is also a limiting factor in the biosynthesis of steroid hormones (Payne and Hales, 2004), our results suggest that StAR may be a target for PFDoA activity in Leydig cells.

Effect of 22R-HC on Progesterone Production

To further examine whether PFDoA inhibits steroidogenesis by disrupting cholesterol transfer, mLTC-1 cells were treated with 100μM PFDoA for 24 h and then incubated with 50μM of the hydrosoluble form of cholesterol, 22R-HC, for 3 h. 22R-HC readily enters the inner mitochondrial membrane without the help of StAR. Treatment with 22R-HC results in a significant 273-fold increase in progesterone levels relative to untreated cells ($p < 0.01$, $n = 6$; Fig. 5). However, PFDoA did not affect 22R-HC–stimulated progesterone production in mLTC-1 cells (22R-HC–treated cells vs. 22R-HC/PFDoA-treated cells: 1116.6 ± 48.9 vs. 1032.4 ± 70.3 ng/mg protein, $n = 6$), indicating that the site of PFDoA activity is cholesterol transfer and that PFDoA does not act on P450scc or the

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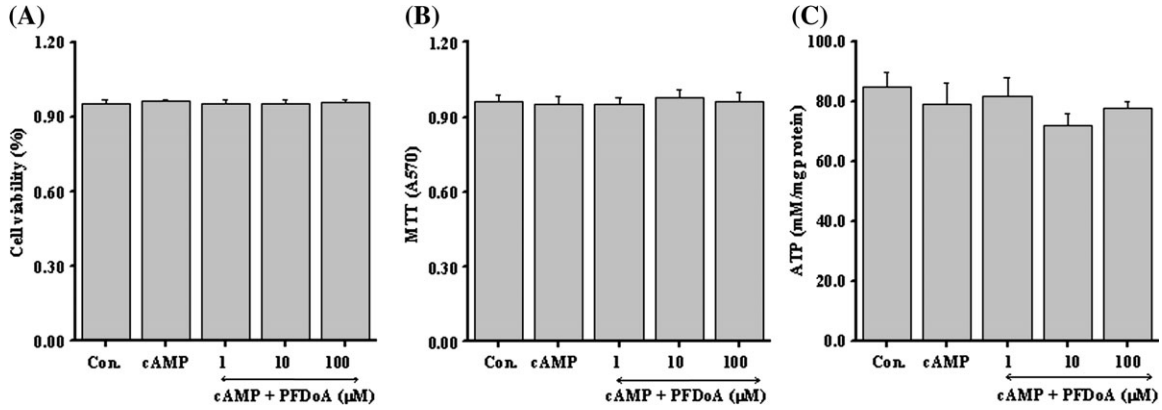

FIG. 2. Effects of PFDoA on cell viability and ATP levels in cAMP-stimulated mLTC-1 cells. Following 3 h of cAMP stimulation, mLTC-1 cells were treated with PFDoA for 24 h. Data are presented as means ± SEM ($n = 6$) from three independent experiments. (A) Viability of mLTC-1 cells; (B) MTT proliferation assay of mLTC-1 cells; and (C) ATP levels of mLTC-1 cells.

other steroidogenic enzymes associated with progesterone synthesis.

Effect of PFDoA on StAR Promoter Activity in mLTC-1 Cells

Because changes in StAR promoter activity can often affect StAR expression, we evaluated the effect of PFDoA on StAR promoter activity. The results show that 100μM PFDoA inhibited significantly StAR promoter activity in mLTC-1 cells ($p < 0.05$) (Fig. 6).

Effects of PFDoA on Mitochondrial ROS and H_2O_2 Levels in mLTC-1 Cells

We then evaluated the levels of ROS and H_2O_2 in PFDoA-treated mLTC-1 cells (Table 1). cAMP did not affect mitochondrial ROS and H_2O_2 levels in mLTC-1 cells. However, treatment with 10 and 100μM PFDoA resulted in mitochondrial ROS levels that were increased 212 and 289%, respectively, relative to cAMP-treated cells ($p < 0.05$, $n = 6$) and H_2O_2 levels that were increased 32 and 55%, respectively, relative to cAMP-treated cells ($p < 0.05$).

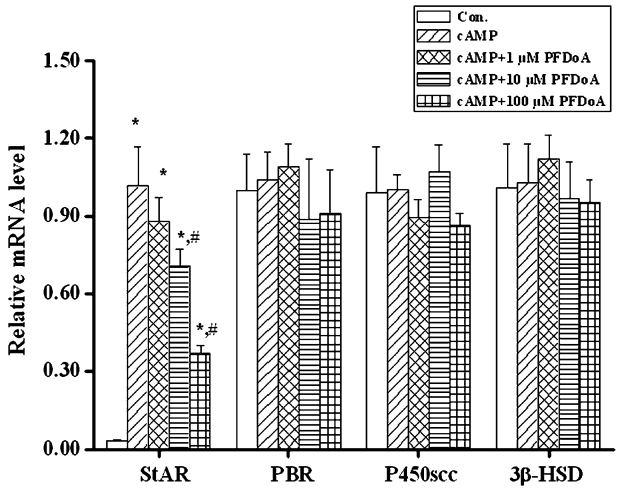

FIG. 3. Effects of PFDoA on the expression of steroidogenesis-related genes in cAMP-treated mLTC-1 cells. Following 3 h of cAMP stimulation, mLTC-1 cells were treated with PFDoA for 24 h. The data are presented as means ± SEM ($n = 3$) from three independent experiments. *significant difference from untreated controls ($p < 0.01$) and #significant difference from cAMP group ($p < 0.05$).

Effects of MnTMPyP on ROS and Progesterone Production in PFDoA-Treated mLTC-1 Cells

In order to determine whether ROS mediate PFDoA-induced inhibition of steroidogenesis in mLTC-1 cells, MnTMPyP, a superoxide dismutase analog, was used to eliminate excessive ROS in the cells. The cells were incubated with MnTMPyP and PFDoA for 24 h and then treated with cAMP for 3 h. The results show that 25 and 50μM MnTMPyP significantly decreased mitochondrial ROS and H_2O_2 levels in mLTC-1 cells treated with 100μM PFDoA ($p < 0.05$, $n = 6$; Table 2). Moreover, 50μM MnTMPyP restored ROS and H_2O_2 levels to control levels. In addition, 25 and 50μM MnTMPyP markedly increased the amount of progesterone in mLTC-1 cells treated with 100μM PFDoA. However, 50μM MnTMPyP did not fully restore progesterone levels in PFDoA/cAMP-treated cells to the level of cAMP-treated cells ($p < 0.05$, $n = 6$). These results show that ROS and H_2O_2 are partially involved in PFDoA-induced steroidogenesis in cAMP-stimulated mLTC-1 cells.

Effect of MnTMPyP on StAR Expression in PFDoA-Treated mLTC-1 Cells

MnTMPyP (25 and 50μM) markedly increased StAR mRNA and protein levels in mLTC-1 cells treated with 100μM PFDoA ($p < 0.05$, $n = 3$; Fig. 7). However, 50μM MnTMPyP only restored StAR mRNA and protein levels to 79 and 73% of those in cAMP-treated cells, respectively. Significant differences in StAR mRNA and protein levels were

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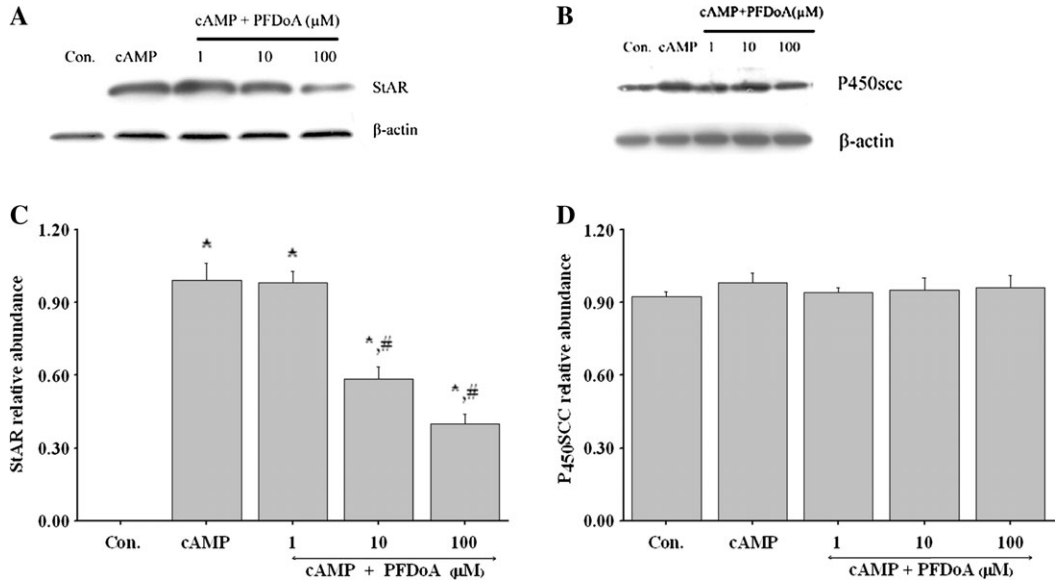

FIG. 4. Effects of PFDoA on StAR and P450scc protein levels in cAMP-treated mLTC-1 cells. Following 3 h of cAMP stimulation, mLTC-1 cells were treated with PFDoA for 24 h. A and B show representative immunoblots of the 30-kDa StAR and 49-kDa P450scc proteins, respectively. C and D show the quantification of StAR and P450scc protein levels, respectively. The data are presented as means ± SEM ($n = 3$) from three independent experiments. *significant difference from untreated controls ($p < 0.01$) and #significant difference from the cAMP group ($p < 0.01$).

observed between cAMP- and MnTMPyP/cAMP/PFDoA-treated cells. Taken together, these results suggest that MnTMPyP can partially rescue the PFDoA-induced inhibition of StAR expression in cAMP-stimulated mLTC-1 cells.

Effect of H_2O_2 on Steroidogenesis in cAMP-Stimulated mLTC-1 Cells

To examine the role of ROS in regulating steroid synthesis, mLTC-1 cells were treated with H_2O_2 and 1mM cAMP for 3 h. The results from this analysis show that 250μM H_2O_2 inhibited progesterone production (Supplementary figure 1) but did not influence the StAR promoter activity (Fig. 6) or StAR expression at mRNA and protein levels in mLTC-1 cells (data not shown).

DISCUSSION

In the present study, PFDoA exhibited a dose-dependent inhibitory effect on progesterone or testosterone production in cAMP-stimulated mLTC-1 cells or rat primary Leydig cells, respectively. cAMP-induced StAR expression was also

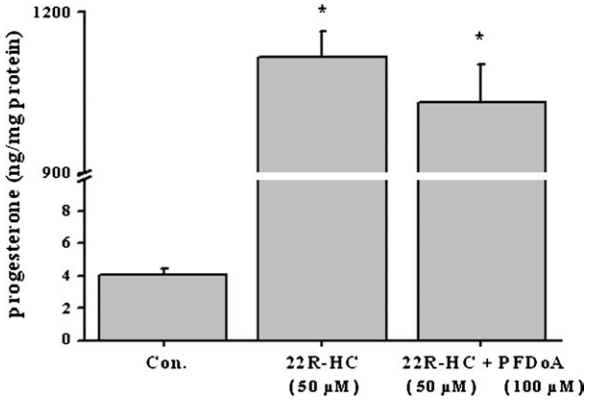

FIG. 5. Progesterone production in mLTC-1 cells treated with 22R-HC. mLTC-1 cells were treated with 100μM PFDoA for 24 h and then stimulated with 50μM 22R-HC for 3 h. The data are presented as means ± SEM ($n = 6$) from three independent experiments. *significant difference from controls ($p < 0.01$).

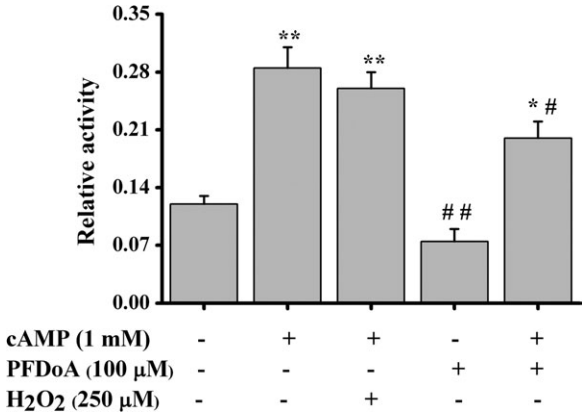

FIG. 6. Effect of PFDoA on StAR promoter activity in mLTC-1 cells. The data are presented as means ± SEM ($n = 3$) from three independent experiments. **significant difference from untreated controls ($p < 0.01$) and #significant difference from the cAMP group (#$p < 0.05$ and ##$p < 0.01$).

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TABLE 1
The Effect of PFDoA on Mitochondrial H_2O_2 and ROS Levels in cAMP-Stimulated mLTC-1 Cells

			cAMP + PFDoA (μM)		
	Control	cAMP	1	10	100
ROS[a]	110.7 ± 2.7	120.8 ± 4.3	118.6 ± 4.8	234.8 ± 3.8*#	320.9 ± 9.6*#
H_2O_2[b]	36.9 ± 1.3	39.4 ± 1.3	40.3 ± 1.8	48.9 ± 1.2*#	57.3 ± 1.2*#

Note. mLTC-1 cells were treated with the indicated concentration of PFDoA for 24 h and then stimulated with 1mM cAMP for 3 h. The levels of mitochondrial ROS and H_2O_2 were normalized to total protein concentrations for each sample and are presented as means ± SEM ($n = 6$) from three independent experiments.

[a]Fluorescence units per microgram protein.

[b]Picomole per milligram protein per minute.

*Significant difference from untreated controls ($p < 0.01$).

#Significant difference from the cAMP group ($p < 0.01$).

inhibited at both mRNA and protein levels following PFDoA exposure. However, PFDoA did not affect mRNA levels of PBR, P450scc, or 3β-HSD in cAMP-stimulated mLTC-1 cells. The studies reported herein confirm and extend earlier observations of the ability of PFAAs to inhibit steroidogenesis in testicular cells (Bookstaff *et al*., 1990; Boujrad *et al*., 2000; Shi *et al*., 2009). In addition, PFDoA also increased levels of ROS and H_2O_2 in mLTC-1 cells. The addition of MnTMPyP to PFDoA-treated Leydig cells partially reversed the inhibition of progesterone production and StAR expression caused by PFDoA and removed excessive ROS and H_2O_2. These results show that ROS and H_2O_2 are associated with PFDoA-induced inhibition of steroidogenesis in cAMP-stimulated mLTC-1 cells.

Chronic PFOA exposure (300 ppm in the diets) in male rats has been shown to produce Leydig cell adenomas associated with increased serum estradiol but did not change in serum testosterone (Biegel *et al*., 2001). These results suggest that testosterone does not modulate the Leydig cell tumor in rats exposed to PFOA. Up to now, there are no reports showing that PFOA exposure causes Leydig cell tumor in mice, indicating that there is a species difference between rat and mouse with respect to the Leydig cell tumor induced by PFAAs. However, this difference does not imply that PFAAs could influence other effects on other activities of Leydig cells between rats and mice, such as steroidogenesis. In this study, PFDoA resulted in similar inhibitory effects on steroidogenesis between rat primary Leydig cells and mLTC-1 cells. Thus, these results show that steroidogenesis can be modulated by PFAAs in both a rat and a mouse model system. The biological significance of these changes remains to be determined.

To recapitulate the functional *in vivo* conditions of Leydig cells, the cells were stimulated with cAMP. PFDoA treatment of Leydig cells for 24 h inhibited cAMP-stimulated steroidogenesis in a dose-dependent manner. These results are similar to those obtained in our previous study in which chronic (110 days) or acute (14 days) exposure to PFDoA inhibited testosterone production in male rats (Shi *et al*., 2007; 2009). These results show that PFDoA possesses an antiandrogen effect in male rats. The trypan blue dye exclusion assay and MTT analysis depend on intact cellular membranes and mitochondrial integrity, respectively (Castedo *et al*., 2002; Mosmann, 1983). These assays can be used as measures of whether cell toxicity is irreversible and obvious (Castedo *et al*., 2002). We did not find significant effects of PFDoA (1–100μM) on cell viability with trypan exclusion or MTT analysis, indicating that PFDoA does not exhibit overt cellular toxicity at these doses and treatment times. Moreover, because ATP is an important indicator for assessing intact cellular activity, the finding that cellular ATP levels were not affected by PFDoA further supports this conclusion (Chandel and Schumacker, 1999). In addition, 24 h of treatment with 10μM PFDoA, followed by washing and further incubation in PFDoA-free medium for 24 h, restored progesterone synthesis. This indicates that PFDoA does not cause irreversible effects on Leydig cell function. However, the reason for the

TABLE 2
The Effect of MnTMPyP on Levels of H_2O_2, ROS, and Progesterone in mLTC-1 Cells

				PFDoA + MnTMPyP (μM)	
	Control	PFDoA (100μM)	MnTMPyP (50μM)	25	50
ROS[a]	107.9 ± 3.3	332.2 ± 12.1*	112.3 ± 3.6	207.3 ± 2.2*#	111.7 ± 3.9#
H_2O_2[b]	37.7 ± 0.9	56.6 ± 1.0*	35.9 ± 1.7	48.8 ± 1.3*#	36.8 ± 1.0#
Progesterone[c]	1079.3 ± 40.0	480.0 ± 19.7*	1112.1 ± 22.5	629.7 ± 49.3*#	741.0 ± 22.7*#

Note. mLTC-1 cells were treated with 100μM PFDoA, 50μM MnTMPyP, or 100μM PFDoA and 50μM MnTMPyP for 24 h, followed by stimulation with 1mM cAMP for 3 h. Control represents cells treated with cAMP alone. The data are expressed as described in Table 1 ($n = 6$).

[a]Fluorescence units per microgram protein.

[b]Picomole per milligram protein per minute.

[c]Nanogram per milligram protein.

*Significant difference from cAMP alone group ($p < 0.05$).

#Significant difference from PFDoA group ($p < 0.05$).

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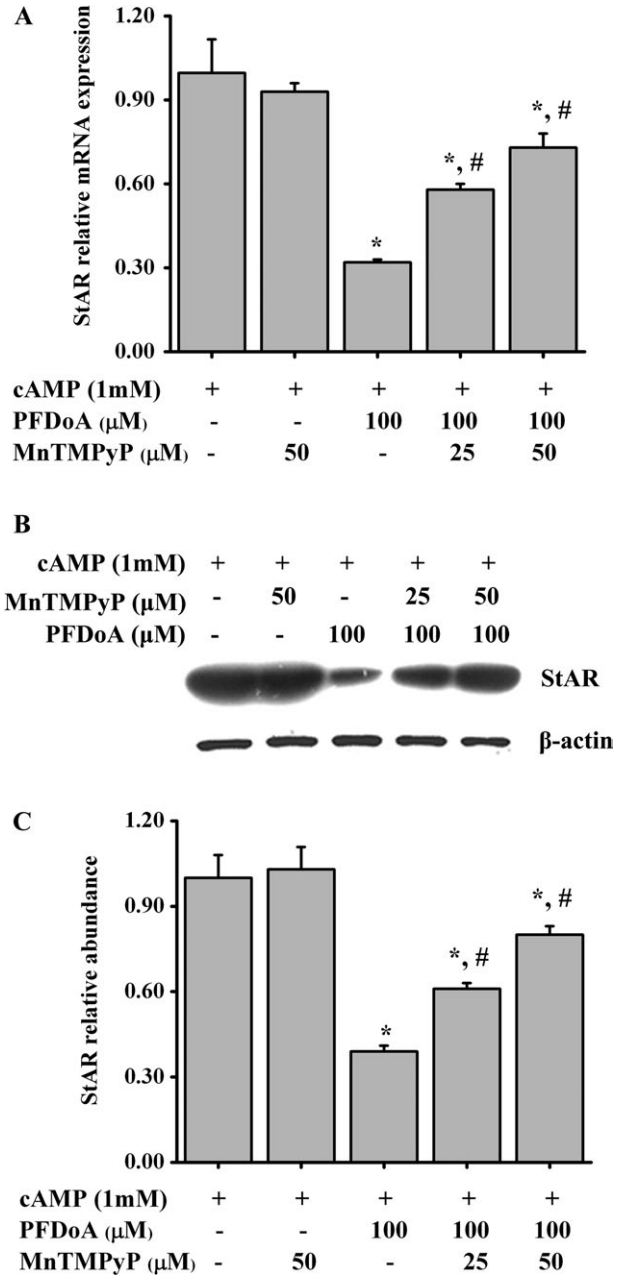

FIG. 7. Effects of MnTMPyP on StAR mRNA and protein levels in mLTC-1 cells. The data are presented as means ± SEM ($n = 3$) from three independent experiments. (A) StAR mRNA; (B) immunoblots of the 30-kDa StAR protein; and (C) quantification of StAR protein levels. *significant difference from the cAMP group ($p < 0.05$) and #significant difference from the cAMP/MnTMPyP group ($p < 0.05$).

recovery of progesterone in these experiments remains uncertain. Taken together, these results suggest that the inhibition of steroidogenesis caused by PFDoA was not due to overt cellular toxicity and may be the consequence of a direct effect on Leydig cells.

Progesterone production includes some key steps in Leydig cells (Payne and Hales, 2004). Cholesterol is first carried from the outer mitochondrial membrane to the inner mitochondrial membrane by StAR and PBR. It is then converted to pregnenolone by P450scc in the inner mitochondrial membrane. Pregnenolone is converted to progesterone by 3β-HSD catalysis in the cytoplasm. Exposure to PFDoA does not inhibit the expression of PBR, P450scc, or 3β-HSD, but it reduces levels of StAR mRNA and protein, suggesting that StAR is particularly sensitive to PFDoA. PFDoA did not affect 22R-HC–stimulated progesterone production in mLTC-1 cells, confirming that PFDoA targets the StAR-mediated step of cholesterol transport but not other steps in Leydig cell steroidogenesis. StAR-mediated transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane in Leydig cells is considered the rate-limiting step in steroidogenesis (Payne and Hales, 2004). Some studies have demonstrated that the inhibition of StAR mRNA or protein levels results in a dramatic decrease in steroid biosynthesis (Stocco and Clark, 1996). In the present study, we observed StAR inhibition at both the mRNA and the protein levels may have disrupted cholesterol delivery across the mitochondrial membrane, resulting in decreased progesterone synthesis in Leydig cells. Taken together, our results provide further confirmation that the inhibition of StAR mRNA and protein plays a key role in PFDoA-induced inhibition of steroidogenesis in cAMP-stimulated Leydig cells. In our previous studies, in male rats that were exposed to PFDoA for 110 days, we observed obvious StAR inhibition at both the mRNA and the protein levels but no inhibition of other factors in the steroidogenesis pathway (Shi *et al.*, 2009). The results reported herein are similar to our previous results, further confirming that our model is appropriate for investigating the mechanism of PFDoA activity in the testes. In MA-10 Leydig tumor cells, although PFDA inhibits hCG-stimulated steroidogenesis through disruption of cholesterol transport, it inhibits the mRNA and protein levels of PBR but not StAR. This indicates that PFDA suppresses steroidogenesis in MA-10 Leydig tumor cells by abrogating PBR expression (Boujrad *et al.*, 2000). This difference in effect between PFDoA and PFDA may be related to the different cell line examined (PFDoA: mLTC-1 cells vs. PFDA: MA-10 cells). These two results suggest that PFAAs with different carbon chain lengths may disrupt steroidogenesis via different molecular mechanisms. Indeed, carbon chain length may mediate the toxic effects of PFAAs. For example, PFDoA increased serum cholesterol levels in male rats (Shi *et al.*, 2007; 2009), while PFOA and PFOS (eight carbons) reduced serum cholesterol levels in rodents (Lau *et al.*, 2007; Pastoor *et al.*, 1987). Moreover, PFAAs possess different pharmacokinetic properties, with longer chain PFAAs generally exhibiting greater accumulation in the body relative to shorter chain PFAAs (Lau *et al.*, 2007; Kudo and Kawashima, 2003b). Thus, differences in carbon chain length may be served as another explain for the different sites of activity of PFDoA and PFDA in Leydig cells.

In order to further investigate the mechanism by which PFDoA disrupts StAR expression, we examined the effects of

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PFDoA on StAR promoter activity. PFDoA significantly inhibited StAR promoter activity, indicating that PFDoA may interact with the StAR promoter. The specific mechanisms responsible for the PFDoA-induced decrease in StAR promoter activity remain to be determined.

Exposure to environmental pollutants often results in increased levels of ROS and an oxygen stress response in cells (Monroe and Halvorsen, 2009). Some studies have shown that ROS, such as superoxide anions and H_2O_2, inhibit steroidogenesis *in vitro* (Diemer *et al*., 2003; Tsai *et al*., 2003). ROS include oxygen radicals, such as superoxide anions and hydroxyl radicals, and reactive oxygen and nitrogen species, such as H_2O_2, nitric oxide, and peroxynitrite anions (Turner *et al*., 1997). ROS such as superoxide anions and H_2O_2 play important roles in endocrine disruption by environmental compounds and have been known to inhibit testicular steroidogenesis (Turner *et al*., 1997; Zini and Schlegel, 1997). In the present study, PFDoA treatment for 24 h resulted in increased levels of mitochondrial ROS and H_2O_2 and the inhibition of steroidogenesis in mLTC-1 cells. After excessive ROS and H_2O_2 were removed by MnTMPyP, a partial recovery of progesterone production was observed in PFDoA-treated cells. These results show that ROS are important factors in mediating the suppression of steroidogenesis in Leydig cells exposed to PFDoA. Indeed, ROS reduced levels of mature 30-kDa StAR protein in a dose-dependent fashion in MA-10 cells, indicating that ROS act at the site of cholesterol transfer (Diemer *et al*., 2003; Tsai *et al*., 2003). H_2O_2 and superoxide anions have been shown to decrease StAR protein levels, but not StAR mRNA levels, in MA-10 cells (Diemer *et al*., 2003). Our results showing that MnTMPyP partially restored StAR mRNA and protein levels indicate that ROS are involved in the inhibition of StAR expression in PFDoA-treated cells. Because H_2O_2 is a ROS, it contributes to the inhibition of ROS-induced StAR expression and steroidogenesis in PFDoA-treated Leydig cells. Our observations reveal that in addition to ROS, other factors may participate in the suppression of StAR expression and steroidogenesis in PFDoA-treated Leydig cells. Interestingly, StAR mRNA levels increased after elimination of excess ROS from PFDoA-treated mLTC-1 cells. This result is in contrast to previous reports in which ROS did not affect StAR mRNA levels (Diemer *et al*., 2003). The influence of ROS production during PFDoA treatment on StAR expression requires further detailed examination.

The effects of exogenous H_2O_2 on StAR expression are different from those of endogenous ROS from PFDoA treatment. We speculate that 24-h PFDoA treatment may change the response of mLTC-1 cells to ROS, such as H_2O_2, such that ROS exhibit the inhibitory effects on StAR expression at the mRNA and protein levels, though treating solely with H_2O_2 did not affect StAR expression in this trial. In addition, because ROS are not stable and quickly scavenged in cells, exogenous H_2O_2 may not model endogenous ROS optimally.

The effective dose of PFDoA (10μM) in the current study is higher than the observed upper range of this chemical (0.022 ng/ml) in human serum (Guruge *et al*., 2005). However, there are some differences between rodents and humans. For example, the biological half-life of PFOA in humans was calculated to be about 2.3–3.8 years (Renner, 2003), which was far longer than that in rats (Vanden Heuvel *et al*., 1991). Therefore, it is difficult to perform an accurate comparison of exposure doses between different species, as shown in other pollutant exposure studies (Arteel *et al*., 2008; Rooney, 2007). Because humans and wildlife may be chronically exposed to lower concentrations of PFDoA, further studies with environmentally relevant doses will provide further insight toward understanding the mechanism of PFDoA-induced inhibition of steroidogenesis.

CONCLUSIONS

Our findings demonstrate that PFDoA increased levels of ROS and H_2O_2 in cAMP-stimulated mLTC-1 cells. The elevation of ROS then caused, at least in part, the inhibition of StAR expression at both mRNA and protein levels as well as the inhibition of steroidogenesis. ROS and StAR are implicated as important contributing factors in the suppression of steroidogenesis in PFDoA-treated Leydig cells.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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