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Proteomic analysis for testis of rats chronically exposed to perfluorododecanoic acid

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ABSTRACT

Perfluorododecanoic acid (PFDoA), a ubiquitous contaminant detected in environmental matrices, wildlife, and human blood, has been shown to produce adverse effects on male reproduction in rats. The mechanism of action of PFDoA in testis, however, is not well understood. In the present study, male rats were orally exposed to PFDoA (0.02, 0.2, and 0.5 mg/kg/day for 110 days), and a two-dimensional gel electrophoresis (2-DE) approach was employed to investigate the alteration of protein expression in the testes. Decreased serum progesterone levels were observed. Matrix-assisted laser desorption/ionization (MALDI) tandem time of flight (TOF/TOF) mass spectrometry analysis allowed the unambiguous identification of 40 differentially expressed proteins. These proteins are mainly involved in mitochondrial respiration, oxidative stress, sperm activity, cytoskeleton and intracellular signal transduction. Furthermore, PFDoA led to decreases in activities of superoxide dismutase (SOD), mitochondrial H-ATPase, and cytochrome *c* oxidase as well as to an increase in lipid peroxidation in testes. Our results indicated that these proteins, which are involved in mitochondrial respiratory and antioxidative responses, play important roles in the inhibition of testicular steriodogenesis in response to PFDoA. Our data demonstrate that alterations of multiple pathways may be associated with the toxic effects of PFDoA on testes. SOD and H-ATPase subunit d may be sensitive to PFDoA exposure in testis.

1. Introduction

Perfluoroalkyl acids (PFAAs) are a family of perfluorinated chemicals that consist of a carbon backbone typically 4–14 carbons in length and include perfluorooctanoic acid (PFOA), perfluorooctane sulfate (PFOS), perfluorodecanoic acid (PFDA), and perfluorododecanoic acid (PFDoA). PFAAs, which are highly resistant to degradation, have been used as surfactants in a variety of products such as plasticizers, paints, cosmetics, and fire-fighting foams (Renner, 2001). As a result of their widespread use and extraordinary persistence, these chemicals and their potential precursors have been found in air, water, soil, wildlife, human blood, and breast milk around the globe (Karrman et al., 2007; Lau et al., 2007; Renner, 2001; Tao et al., 2008). For example, the total amount of PFAAs transferred from nursing mothers to breastfeeding infants was approximately 200 ng/day in Sweden and 23.5 ng/kg of bw/day in the USA (Karrman et al., 2007; Tao et al., 2008). Due to the wide distribution of PFAAs, the potential health risks for humans and wildlife has raised scientific and regulatory concern (Lau et al., 2007).

PFAAs are known as peroxisome proliferators that induce liver enlargement, turbulence of hepatic lipid metabolism, and liver adenomas in rodents (Seacat et al., 2003; Vanden Huevel et al., 2006). In addition, PFAAs such as PFOA and PFOS also exert immune toxicity (Yang et al., 2001) and neuron toxicity (Lau et al., 2007) as well as reproductive and developmental toxicity in rodents (Lau et al., 2004). In male rats, PFOA exposure led to notable testicular toxicities, including testis lesions, decreased testosterone levels in both serum and testicular interstitial fluid, and Leydig cell tumors (Jensen and Leffers, 2008; Lau et al., 2004). PFDA disrupted testosterone and progesterone synthesis in both *in vivo* (Bookstaff et al., 1990) and *in vitro* (Boujrad et al., 2000). Our previous work also indicated that PFDoA exposure at 5 mg/kg/day or 10 mg/kg/day for 14 days resulted in testis cell apoptosis and a decline in serum testosterone (T) levels. Moreover, 110 days PFDoA exposure led to significantly decreased testosterone and expressional changes of testicular steroidogenic genes in rats (Shi et al., 2009). These results suggested that testosterone decline may be involved in the pathway of cholesterol transportation and steroidogenesis and that these pathways were disrupted in testes following PFDoA exposure (Shi et al., 2007). In addition, PFAAs with more than seven carbons in their backbone cannot be easily metabolized and excreted from the body; thus, longer carbon chain PFAAs such as PFDoA have higher accumulation capabilities and are more physiologi-

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cally persistent than shorter chain PFAAs (Ohmori et al., 2003). Taken together, these data suggest that PFDoA may exhibit more toxicities than other PFAAs and may exert deleterious effects on testicular function. Thus, global functional analyses to explore the complicated biochemical mechanism of PFDoA in testes are warranted.

Proteomics is a powerful method in toxicology and will provide insight into the mechanisms of toxic compounds (Wetmore and Merrick, 2004). Specifically, proteome analysis provides an effective way to discover the complex effects of toxic chemicals and related biomarkers for risk assessment (Klenø et al., 2004). This method has been used to study the mechanism of liver toxicity following PFOA exposure in rare minnows (Wei et al., 2008), but the investigation of the protein expression profile in testes from rodents exposed to PFAAs has not yet been conducted. In the present study, male rats were treated with PFDoA for 110 days via gavage. A 2-DE approach was employed to examine the alterations in protein expression in the testes from rats following this chronic exposure to PFDoA. In order to further confirm the proteomic results, the enzymatic activities and gene expression changes were analyzed. Serum progesterone levels were measured by chemiluminescence to identify the toxic endpoints of PFDoA exposure. Based on the proteomic analysis together with the biochemical data, we lay the foundation for understanding the underlying mechanisms of PFDoA toxicity.

2. Materials and methods

2.1. Materials

PFDoA (CAS no. 307-55-1, 95% purity) was purchased from Sigma–Aldrich Corp (St. Louis, MO). Phenylmethylsulfonyl fluoride (PMSF), immobilized IPG strips, and dithiothreitol (DTT) were purchased from Amersham Biosciences (USA). All other chemicals and reagents were analytical grade.

2.2. Animals and treatment

Three-week-old male Sprague–Dawley rats were purchased from Weitong Lihua Experimentary Animal Central, Beijing, China. Animals were placed individually in stainless steel cages and maintained in a mass air displacement room with a 12 h light–dark cycle at 20–26 °C with a relative humidity of 40–60%. Animals had access to food and water *ad libitum*. After a 1-week acclimation period, rats were randomly divided into one control and three treatment groups. Each group contained six rats.

PFDoA was dissolved in 0.2% Tween-20 (Beijing Chemical Reagent Co., Beijing, China) in distilled water. The rats in the treatment groups were exposed to PFDoA via oral gavage at doses of 0.02, 0.2, and 0.5 mg PFDoA/kg body weight/day for 110 days using a dosing volume of 6 ml/kg of body weight. Control animals were dosed with 0.2% Tween-20 (vehicle) in the same volume. At the end of the experiment, six 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 testis was frozen immediately in liquid nitrogen and stored at −80 °C until analysis.

2.3. Serum progesterone

Concentrations of serum progesterone were measured by chemiluminescence using commercial kits (Beijing North Institute of Biological Technology, China) according to the manufacture's recommendations.

2.4. Sample preparation and 2-DE

Testis tissues from six rats per group were homogenized in 1 mL lysis buffer (7 M urea, 4% CHAPS, 30 mM Tris, 1.0% DTT, and 1 mM PMSF). The homogenates were centrifuged at 10,000 × *g* for 15 min at 4 °C to remove tissue and cell debris. Protein concentration of the supernatants was determined using a Bradford Kit (Applygen Technologies Inc., China). Equal amounts of protein from samples from six rats per group were pooled into three samples by random selection of two individual rats. Three pooled samples were then used for electrophoresis. Protein samples (200 μg) from control and treatment groups were loaded onto an IPG strip (Immobiline 24 cm DryStrip pH 4–7, GE Healthcare, USA) for simultaneous rehydration. Isoelectric focusing (IEF) was performed with the following voltage-time program: 40 V for 5 h, 100 V for 6 h, 500 V for 1 h, 2000 V for 1.5 h, and 8000 V for a total of 70,000 V h. After being focused, IPG strips were equilibrated for 15 min in 6 M urea, 2% SDS, 1% DTT, 75 mM Tris (pH 8.8), and 30% glycerol. The strips were equilibrated for 15 min in this solution without DTT but with 2.5% iodoacetamide. The strips were placed on a 12% SDS-polyacrylamide gel and then were run for 6 h at 15 °C using a vertical electrophoresis system (GE Healthcare). After electrophoresis, the gels were fixed in 40% (v/v) ethanol and 10% (v/v) glacial acetic acid for 12 h and then transferred to a sensitizing solution containing 30% (v/v) ethanol, 12.6 mM sodium thiosulfate, and 0.83 M sodium acetate for 1 h. The gels were then washed four times for 1 h with deionized water. The gels were placed in a 14.7 mM silver nitrate solution for 1 h and then were washed for 4 min with deionized water. The gels were developed with 0.24 M sodium carbonate and 13.3 mM formaldehyde for 10 min, followed by the addition of 38.4 mM EDTA for 45 min to stop the reaction. Finally, the gels were washed three times with deionized water.

2.5. Spot detection and comparisons

The gels were scanned at a resolution of 600 dpi by the Uniscan D3000 scanner (Tsinghua, China). 2-DE analysis software (ImageMaster 2D platinum, GE Healthcare) was used for protein spot analysis. Protein spots were detected with a combination of automated spot detection and manual interpretation guided by landmark proteins. Protein spot intensity was determined and normalized by calculating the relative spot intensity of the proteins compared to the total intensity of protein in the gel. The differences in spot intensity between control and PFDoA-treated animals were analyzed using the Student's *t*-test. Values of $p < 0.05$ were considered statistically significance.

2.6. Protein identification

The protein spots that were significantly different between control and treatment groups were excised from silver stained gels using a sterile scalpel. After being destained with 50% ACN/25 mM ammonium bicarbonate solution, the gel plugs were dried for 30 min. The dried gel particles were rehydrated for 45 min with trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate at 4 °C, and then incubated at 37 °C for 12 h. After trypsin digestion, the peptide mixtures were extracted in 50% ACN/0.5% TFA and then were dried under the protection of N_2. Finally, the mixtures were analyzed with a 4700 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA). Myoglobin that had been digested by trypsin was used to calibrate the mass instrument with internal calibration mode. All acquired sample spectra were processed using 4700 Explore software (Applied Biosystems) in default mode at Fudan University. Parent mass peaks with a mass range of 700–3200 Da and minimum S/N 20 were chosen for tandem TOF/TOF analysis. The GPS Explorer software (V3.6, Applied Biosystems) was used to submit the combined MS and MS/MS spectra to database search program MASCOT (V2.1, Matrix Science, London, UK). For each spectra search, MS tolerance and MS/MS tolerance were set as 0.2 and 0.6 Da, respectively. One missed tryptic cleavage was allowed. MASCOT protein scores (based on combined MS and MS/MS spectra) of more than 56 were considered statistically significant ($p \leq 0.05$). The individual MS/MS spectra with statistically significant (confidence interval > 95%) best ion score (based on MS/MS spectra) were accepted.

2.7. Lipid peroxidation and enzyme activity

Rat testis was homogenized in PBS (pH 7.4) and was then centrifuged at 3000 × *g*. The supernatant fluid was collected for the analysis of superoxide dismutase activity and lipid peroxidation. Mitochondrial H-ATPase activity was measured according to the reference (Comelli et al., 2007) and the measured activity was expressed as

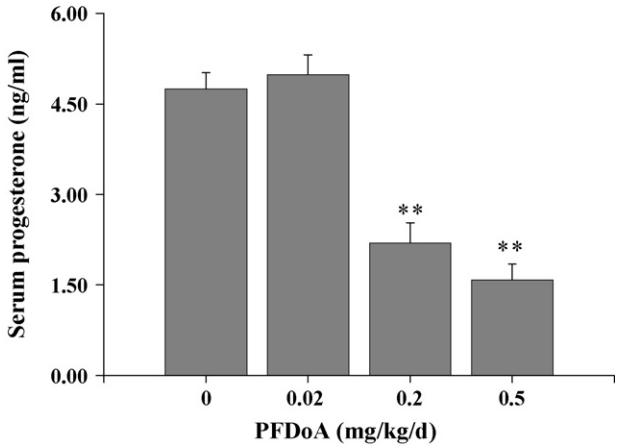

Fig. 1. The effects of chronic PFDoA exposure on serum progesterone levels in male rats. Values represent the average ± S.E.M. from six rats per group. Asterisks indicate a statistically significant difference: $^{**}p < 0.01$.

Table 1
Differentially expressed proteins in testis from control and PFDoA-treated rats.

Spot ID	NCBI	Protein name	Protein score	Sequence[a]	Observed MW/pI	Theoretical MW/pI	Fold change induced by PFDoA (mg/kg/day)[b]		
							0.02	0.2	0.5
Mitochondrial respiratory chain									
1	gi|24233541	Cytochrome *c* oxidase, subunit Va	87	LNDFASAVR	17932/5.98	16119.3/6.08	1.28	1.08	−2.13*
2	gi|55992	Cytochrome *c* oxidase subunit VIa	139	EIMIAAQR GLDPYNMLPPK	13486/6.60	12681.2/6.46	−1.59*	−2.49*	–
3	gi|57164091	Ubiquinol-cytochrome *c* reductase hinge protein	73	EEEEEELVDPLTTVR	11250/4.87	10417/4.9	1.12	−2.33*	−3.35*
4	gi|220904	Subunit d of mitochondrial H-ATP synthase	138	SWNETFHTR LASLSEKPPAIDWAYYR	16547/5.83	18769.6/5.78	−1.97*	−2.32*	−2.21*
Oxidative stress									
5	gi|203658	Cu–Zn superoxide dismutase	258	DGVANVSIEDR VISLSGEHSIIGR GDGPVQGVIHFEQK	14856/5.95	15699.7/5.88	−1.16	−2.48*	−2.93*
6	gi|13027384	Indoleamine 2,3-dioxygenase	58		47032/6.10	45801.6/6.17	2.18*	1.48	2.32*
7	gi|13162287	D-Dopachrome tautomerase	94	FLTEELSLDQDR	12035/6.11	13124.8/6.09	1.24	−1.85*	−2.56*
8	gi|6978859	Ferritin, heavy polypeptide 1	64	YFLHQSHEER	23043/5.73	21086.2/5.62	−2.54*	−1.07	−2.03*
9	gi|62079189	Glyoxalase domain containing 4	199	FQTVHFFR LELQGIQGAVDHSAAFGR	32149/5.10	33246.5/5.11	1.15	−1.19	−1.93*
Sperm activity									
10	gi|13928824	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	70		30683/4.49	29103.3/4.55	−1.07	1.33	−3.24*
11	gi|13242320	A-kinase anchor protein 4	63	MSDDIDWLHSR	90983/6.58	93433.9/6.37	1.32	1.25	−2.97*
12	gi|16924002	DJ-1 protein	101	GAEEMETVIPVDIMR	17133/6.53	19961.4/6.32	1.32	−1.13	−2.13*
13	gi|8393910	Phosphatidylethanolamine binding protein 1	80	FKVESFR	22751/5.53	20788.4/5.48	−2.13*	–	−3.24*
14	gi|10863989	Acid phosphatase 1, soluble isoform A	71	SPIAEAVFR	24137/6.14	18139.8/6.08	1.04	2.34*	2.60*
15	gi|71795633	C-src tyrosine kinase	72		51369/6.8	50713.8/6.62	1.2	−1.79*	−3.32*
16	gi|55391508	Albumin	143	LVQEVTDFAK FKDLGEQHFK	65793/6.23	68714.1/6.09	1.03	−1.57*	−2.13*
Cytoskeleton reorganization									
17	gi|57527565	Rho, GDP dissociation inhibitor (GDI) beta	67		18832/4.95	22869.5/4.99	−1.43	−1.17	3.52*
18	gi|56912233	Keratin complex 1, acidic, gene 14	67		50357/5.03	52650.9/5.08	1.25	1.32	−2.14*
19	gi|8393696	Stathmin 1	166	ASGQAFELILSPR	15369/5.71	17277.9/5.76	1.11	−1.29	−1.69*
20	gi|30842809	Glia maturation factor, gamma	100		17035/5.49	16768.5/5.56	1.27	1.75*	−1.25
21	gi|57012436	Keratin 10	58		61035/5.23	56470.5/5.1	1.17	−1.22	−2.08*
22	gi|56847618	Type I keratin KA16	74		51579/5.11	50745.7/5.05	1.02	−1.58*	1.12
Protein quality control									
23	gi|12084772	Chain A, crystal structure of rat alpha 1-Macroglobulin receptor binding domain	83		14257/5.71	15353.8/5.78	1.19	−1.30	−1.55*
24	gi|109471020	Similar to proteasome inhibitor PI31 subunit isoform 1 (predicted)	64		27358/4.93	29835.9/5.08	−1.87	−1.08	−1.51*
25	gi|16758810	Ubiquitin-conjugating enzyme E2N	306	SNEAQAIETAR	15948/6.31	17113/6.13	−1.63	−2.10*	−3.33*
26	gi|61557351	COP9 (constitutive photomorphogenic) homolog subunit 8	72		21758/5.04	23221/5.09	1.34	−1.16	−1.92*
Signal transduction									
27	gi|8393962	Phosphatidylinositol transfer protein	63	HVEAIYIDIADR	29368/5.94	31887.1/5.97	−1.12	−1.27	−2.55*
28	gi|4139938	Chain A, recombinant rat annexin V, W185a mutant	158	GLGTDEDSILNLLTAR	33790/5.01	35607.2/4.93	1.13	−2.3*	−1.21
29	gi|72255531	EF hand domain containing 2	94		27847/5.05	26742.5/5.01	1.23	−1.14	−1.93*
30	gi|14091736	Inositol (myo)-1(or 4)-monophosphatase 1	84		28936/5.31	30491.4/5.17	−1.09	−1.30	−1.43*

Table 1 (*Continued*)

Spot ID	NCBI	Protein name	Protein score	Sequence[a]	Observed MW/pI	Theoretical MW/pI	Fold change induced by PFDoA (mg/kg/day)[b]		
							0.02	0.2	0.5
31	gi|62658198	Similar to Ran-specific GTPase-activatin protein (predicted)	179	FLNAENAQK VAEKLEALSVR FASENDLPEWK TLEEDEEELFK	20932/5.23	23581.7/5.15	1.21	−1.07	−1.47*
32	gi|6840951	PKCq-interacting protein PICOT	75	HNIQFSSFDIFSDEEVR	29856/4.80	31341/4.9	1.52	1.13	−2.12*
Other functions									
33	gi|11120730	Fragile histidine triad	97	HVHVHILPR	21346/5.97	17336.8/6.16	1.24	1.56*	1.32
34	gi|71051822	Unknown (protein for MGC:116262)	69	YEELQITAGR	61368/6.90	59212.7/8.06	−1.13	−1.46*	1.09
35	gi|62656599	Similar to intraflagellar transport protein IFT20 (predicted)	88		14078/5.12	15259/5.07	1.34	1.12	−2.05*
36	gi|203078	Nucleolar protein B23.2	119	VDNDENEHQLSLR	29032/4.65	28367.7/4.55	1.23	−1.23	−2.5*
37	gi|47059185	Divalent cation tolerant protein CUTA	66	TQSSLVPALTEFVR	17035/6.33	16402.6/6.29	1.23	1.06	−1.72*
38	gi|20663827	Chain A, rat transthyretin complex with thyroxine	73	ALGISPFHEYAEVVFTANDSGHR	12083/5.65	13589.8/5.76	1.35	−1.49	−2.78*
39	gi|68341965	N-terminal Asn amidase	100		35089/5.91	34579.7/5.84	−1.10	1.74*	1.22
40	gi|90296198	Liver cancer-related protein	205	LGPYNEELR IHVIDHSGVR LSEEEAQASAISVGSR	28746/5.06	27165.2/4.98	1.07	−1.10	−1.66*

[a] The sequence of matched peptides identified by MS/MS (ion cross confidence interval CI % >90%).
[b] Fold changes were calculated by comparing the intensity of protein between control and treatment samples ($n = 3$).
* $p < 0.05$.

Fig. 2. Representative 2-DE image for protein expression maps from rat testis (silver stained-gel). Spots of proteins that exhibited statistically significant differences between control and PFDoA-treated rats are indicated with a red ring and numeral. Each protein is annotated in Table 1.

nmol of ATP per min per mg of protein (nmol ATP/min/mg protein). The levels of LPO, SOD activity, and mitochondrial cytochrome *c* oxidase (COX) activity were measured in the samples using a commercial LPO and SOD kit (Jiancheng Bio-technology Inc., Nanjing, China) and COX kit (Genmed Scientifics Inc., USA) according to the manufacturer's recommendations.

2.8. RNA extraction and analysis of gene expression

Total testis RNA was extracted using the TRIZOL reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The cDNA was then synthesized via reverse transcription (RT) using an oligo-$(dT)_{15}$ primer (Promega, Madison, WI) and the M-MuLV reverse transcriptase (Promega, Madison, WI) in accordance with the manufacturers' recommendations. Real-time PCR reactions were performed with the Stratagene Mx3000P qPCR system (Stratagene, La Jolla, CA). SYBR Green PCR Master Mix reagent kits (Takara, Dalian, China) were used according to the manufacturer's instructions for quantification of gene expression. Rat-specific primers were designed for the genes of interest (Supplementary Data). The housekeeping gene β-actin was used as an internal control. The Ct value was obtained in the amplification reaction. Ct represents the cycle at which the fluorescence signal is first significantly different from background. The relative expression ratio (R) of a target gene was calculated based on the description of Barlow et al. (2003).

2.9. Statistical analysis

For the analysis of protein intensity in 2-DE, fold changes were calculated by comparing the control and treatment groups. The significant differences between the protein expression of the control and treatment groups was analyzed as described above. For serum progesterone level, lipid peroxidation, enzyme activ-

ity, and gene expression, the raw data were analyzed using SPSS for Windows 13.0 Software (SPSS, Inc., Chicago, IL). All values are expressed as average ± S.E.M. Differences between the control and treatment groups were determined by Dunnett's post hoc two-sided *t*-test. Differences were considered significant when the probability was less than 0.05 ($p < 0.05$).

3. Results

3.1. Serum progesterone levels

The effects of chronic PFDoA exposure on serum progesterone levels in male rats were analyzed (Fig. 1). Following 110 days of PFDoA treatment, the serum progesterone levels were significantly decreased by 53% and 67% in the 0.2- and 0.5-mg-dosed rats, respectively ($p < 0.01$).

3.2. Protein profiles

Following PFDoA exposure of rats, testes samples were analyzed via a 2-DE approach to investigate the alteration of protein expression in the testes. Approximately 600 protein spots were detected in the 2-DE and 374 spots were successfully matched across the whole set of images. Comparison of the intensities of matched protein spots in the control and treatment groups revealed 65 differentially expressed spots ($p < 0.05$). We successfully identified 40 proteins using MS analysis (Fig. 2). The differentially expressed proteins in the rat testes from the control and treatment groups are listed in Table 1 . Four spots were identified as fragments of enzymes related to the mitochondrial respiratory chain. These proteins were cytochrome *c* oxidase subunit Va (COX5a) and subunit Via (COX6a), ubiquinol-cytochrome *c* reductase hinge protein (UQCRH), and subunit d of mitochondrial H-ATPase. Among these proteins, for animals exposed to PFDoA at 0.2 and 0.5 mg/kg/day, UQCRH expression was significantly decreased by 2.33- and 3.35-fold, respectively. The protein levels of COX6a and subunit d of mitochondrial H-ATPase were markedly reduced by PFDoA at all doses. In addition, some proteins involved in oxidative stress exhibited significant differences between the control and treatment groups. For example, compared to the control rats, SOD levels were significantly decreased by 2.48- and 2.93-fold at 0.2- and 0.5-mg-dosed rats, respectively. However, the levels of indoleamine 2,3-dioxygenase (IDO) was actually upregulated by PFDoA at the 0.02 and 0.5 mg/kg/day doses. Furthermore, 110 days of PFDoA exposure also led to altered expression of sperm activity-related proteins. For example, compared to the control, the levels of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHA) and A-kinase anchor protein 4 (AKAP4) were significantly decreased by 3.24- and 2.97-fold, respectively, in the highest dose group. Changes in several cytoskeletal proteins such as stathmin 1 were noted. In addition, changes in expression of proteins that participate in protein degradation, cell signaling transduction, lipid transfer, copper homeostasis, and protein modification were also observed in certain dose group.

3.3. Enzyme activity and lipid peroxidation

To further confirm that alterations in expression of enzymes involved in the mitochondrial respiratory chain and oxidative stress identified via our proteomic method, the related enzyme activities were measured. Indeed, PFDoA exposure significantly decreased mitochondrial COX activity in rat testes in a dose-dependent manner (Fig. 3D). This enzyme activity was significantly decreased by

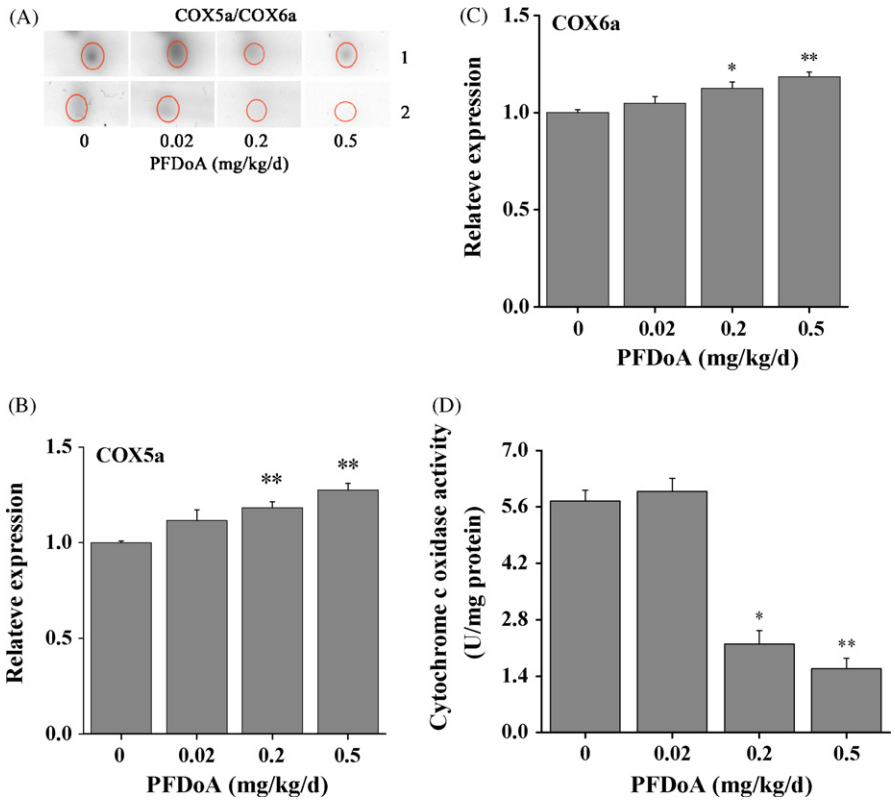

Fig. 3. Protein expression and mRNA levels of cytochrome *c* oxidase subunit Va (COX5a) and cytochrome *c* oxidase subunit VIa (COX6a), and enzymatic activity of cytochrome *c* oxidase in testis of rats exposed to PFDoA. (A) Changes in protein spots 1 and 2 in the 2-DE (Table 1). (B and C) Changes in mRNA levels. (D) COX activity levels. Values represent the average ± S.E.M. from six rats per group. Asterisks indicate a statistically significant difference: $^{*}p < 0.05$, $^{**}p < 0.01$.

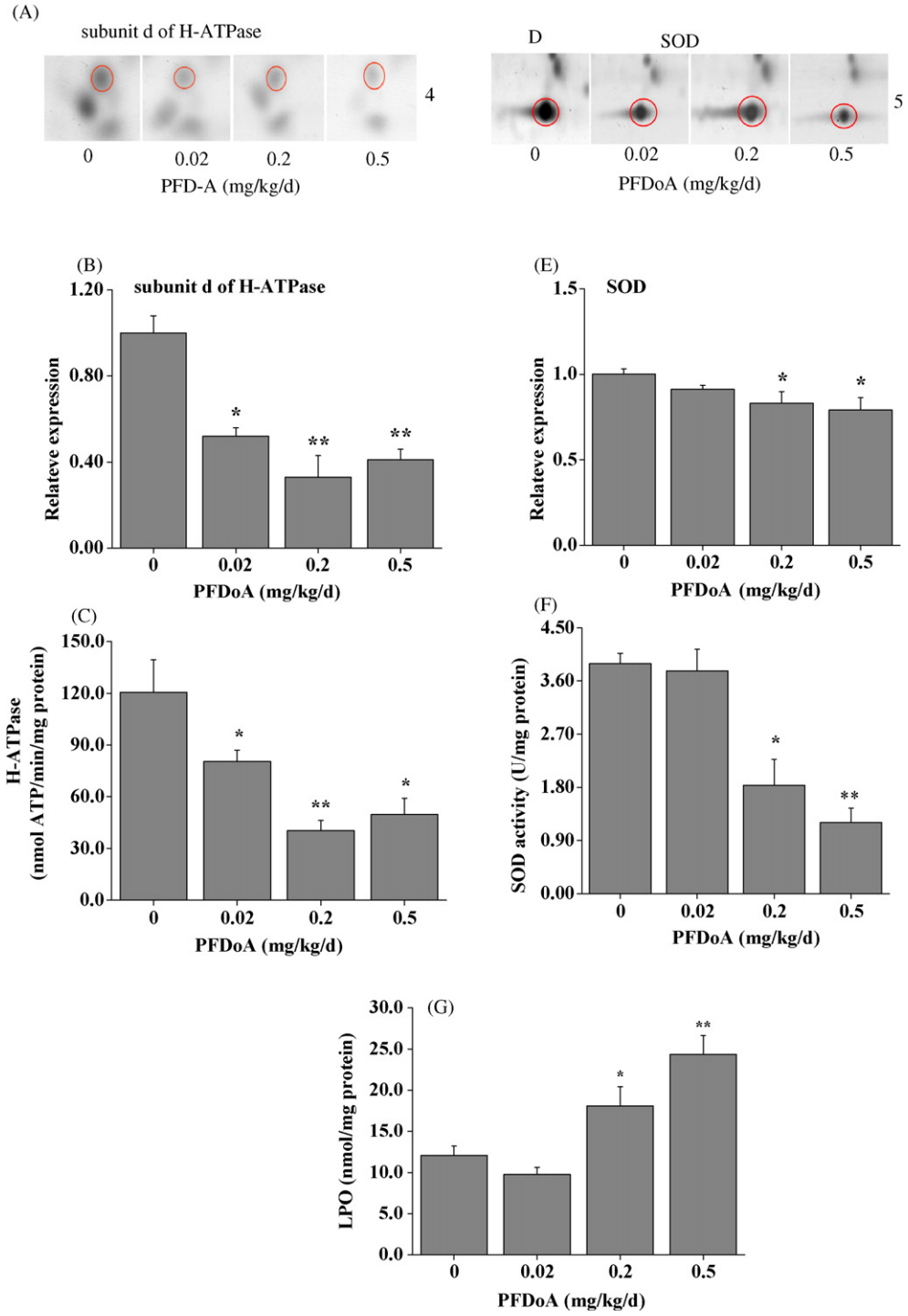

Fig. 4. Protein expression and mRNA levels of H-ATPase subunit d and superoxide dismutase (SOD), and enzymatic activity of H-ATPase and SOD in testis of rats exposed to PFDoA. (A) Changes in protein spot 4 (H-ATPase subunit d; see Table 1). (B) Changes in mRNA levels of H-ATPase. (C) H-ATPase activity. (D) Changes in protein spot 5 (SOD; Table 1). (E) Changes in mRNA levels of SOD. (F) SOD activity. (G) Testicular lipid peroxidation (LPO) levels. Values represent the average ± S.E.M. from six rats per group. Asterisks indicate a statistically significant difference: $^{*}p < 0.05$, $^{**}p < 0.01$.

62% and 73% compared to the control at doses of 0.2 ($p < 0.05$) and 0.5 mg/kg/day ($p < 0.01$), respectively. H-ATPase activity was markedly inhibited by PFDoA at all three dosages ($p < 0.05$; Fig. 4C). SOD activity in testes tissues was also markedly decreased by 53% and 69% in the 0.2-mg-dosed ($p < 0.05$) and 0.5-mg-dosed rats ($p < 0.01$), respectively (Fig. 4F). A significant increase in the level of LPO in rat testes was observed in the 0.2-mg- or 0.5-mg dosed groups ($p < 0.05$; Fig. 4G).

3.4. Gene expression

Based on the proteomic results, we examined the expression of genes related to mitochondrial respiratory chain, oxidative stress, and sperm activity to compare the correlation between protein expression and gene expression (Figs. 3–5). Expression of COX5a (Fig. 3B), COX6a (Fig. 3C), and AKAP4 (Fig. 5H) in testes was significantly increased in the 0.02- and 0.5-mg-dosed groups. The

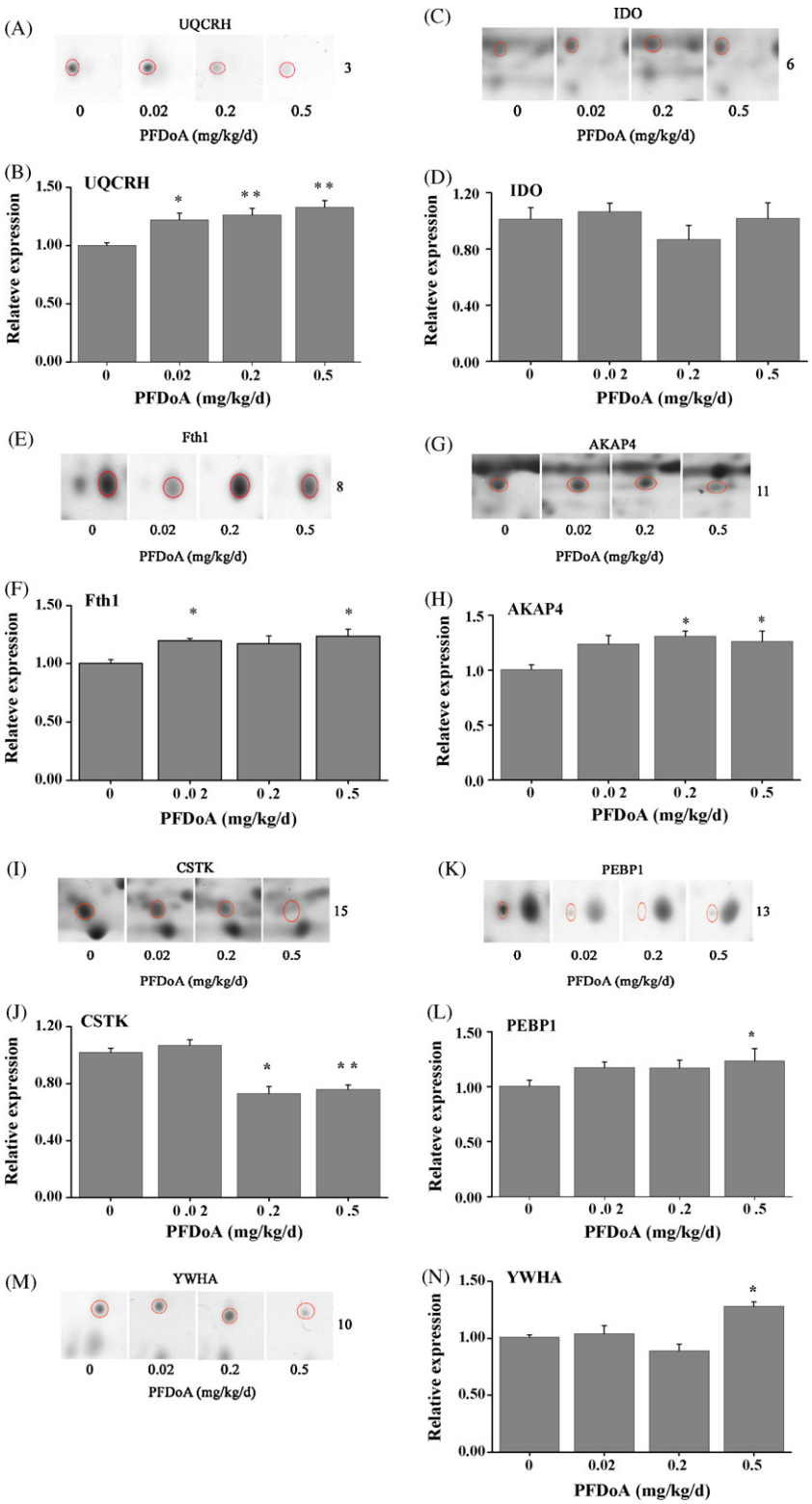

Fig. 5. (A–N) Effects of chronic PFDoA exposure on ubiquinol-cytochrome *c* reductase hinge protein (UQCRH), indoleamine 2,3-dioxygenase (IDO), ferritin heavy polypeptide 1 (Fth1), A-kinase anchor protein 4 (AKAP4), c-src tyrosine kinase (CSTK), phosphatidylethanolamine binding protein 1 (PEBP1), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHA) expression at both the protein and mRNA levels in rat testes. Protein changes are further described in Table 1. Values represent the average ± S.E.M. from six rats per group. Asterisks indicate a statistically significant difference: $^{*}p < 0.05$, $^{**}p < 0.01$.

UQCRH mRNA level was markedly increased by PFDoA at all doses (Fig. 5B), and the mRNA levels of H-ATPase subunit d was significantly reduced (Fig. 4B). Obvious downregulation of SOD expression was observed for the 0.02- and 0.5-mg-dosed groups (Fig. 4E). Although the mRNA levels of ferritin heavy polypeptide 1 (Fth1) were significantly increased at doses of 0.2 and 0.5 mg PFDoA/kg/day compared to vehicle treatment (Fig. 5F), no marked differences in the mRNA levels of IDO were exhibited between any groups (Fig. 5D). In addition, expressions of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein

(YWHA) (Fig. 5N) and phosphatidylethanolamine binding protein 1 (PEBP1) (Fig. 5L) were elevated by PFDoA at the highest dose. The mRNA level of c-src tyrosine kinase (CSTK) was markedly decreased in the 0.02- and 0.5-mg-dosed groups compared to the control group (Fig. 5J).

4. Discussion

In the present study, decreased serum progesterone levels were observed. Furthermore, 40 differentially expressed proteins were identified in the testes of rats exposed to PFDoA using the proteomic method. These proteins are primarily involved in mitochondrial respiratory chain, oxidative stress, and sperm activity, as well as cytoskeleton, protein quality control, and intracellular signal transduction. In addition, PFDoA not only altered the activities of SOD, mitochondrial H-ATPase, and COX but also altered the LPO levels in testes of treated animals. Alterations in mRNA levels of some genes involved in sperm activity, mitochondrial function, and oxidative stress were also detected in testes of rats exposed to PFDoA. These results firstly demonstrate that multiple pathways are important for the testicular toxicity of PFDoA using proteomic analysis.

In this study, a significant decline in the levels of serum progesterone, a precursor for testosterone synthesis, was observed at 0.2 and 0.5 mg/kg/day. This decreased progesterone provides an explanation for the testosterone decline in the same animals treated with PFDoA at 0.2 and 0.5 mg PFDoA/kg/day for 110 days in the previous study (Shi et al., 2009). In addition, 14 days PFDoA exposure led to the decline in serum testosterone levels in male rats (Shi et al., 2007). These results clearly demonstrate that PFDoA disrupts steroidogenesis in male rats.

In the proteomic analysis, we found that four proteins involved in the mitochondrial respiratory chain were significantly altered by PFDoA. UQCRH plays an important role in the electron transfer steps of the respiratory chain by facilitating cytochrome *c*1 and cytochrome *c* interaction (Modena et al., 2003). Reduction of UQCRH protein levels in the 0.2 and 0.5 mg PFDoA/kg/day groups implies that PFDoA may disrupt mitochondrial electron transfer. COX is responsible for generating a proton gradient across the inner mitochondrial membrane through performing the reduction of O_2 to H_2O_2 (Brzezinski and Gennis, 2008). H-ATPase initiates ATP synthesis in mitochondria by driving proton gradients (Weber, 2006). Furthermore, COX and H-ATPase functions have been shown to be dependent on their intact subunits (Weber, 2006). Therefore, the reduction in protein levels of COX5a/COX6a and subunit d of H-ATPase following PFDoA exposure implies that PFDoA not only disrupts mitochondrial proton gradient formation but also inhibits mitochondrial energy production. The significant reduction of COX and H-ATPase activity at all three dosages also further supports the proteomic results of decreased COX5a/COX6a and H-ATPase subunit d. This evidence indicates that PFDoA affects mitochondrial function possibly through disrupting the mitochondrial respiratory chain and energy formation in testes. Since the normal mitochondrial respiration and energy production are essential to produce progesterone in Leydig cells (Allen et al., 2006), decline of UQCH, COX5a/COX6a, and H-APTase subunit d at protein level may play important roles in decreased progesterone. In addition, though PFOA and PFDA may affect mitochondrial respiration in isolated liver cells, these two PFAAs stimulate the mitochondrial energy production (Langley, 1990; Starkov and Wallace, 2002; Kleszczyński et al., 2009). Moreover, PFDA stimulated latent H-ATPase activity in isolated rat liver mitochondria (Langley, 1990). Thus, PFDoA may exhibit different effect on mitochondrial energy system from PFOA and PFDA. This discrepancy may be associated with the different tissue (testis vs. liver), the different experiment type (*in vivo* vs. *in vitro*) and the different structure between PFDoA and PFOA/PFDA.

Abnormal mitochondrial respiration produces abundant reactive oxygen species (ROS). If these ROS cannot be fully cleared by antioxidants, the oxidative stress response will be initiated (Orrenius et al., 2007). In the present study, five antioxidative stress proteins including SOD, Fth1, D-dopachrome tautomerase (DDT), glyoxalase domain containing 4 (Glod4), and IDO were identified by proteomic analysis. The declines of SOD protein in the 0.2- and 0.5-mg-dosed groups indicate that PFDoA disrupts SOD function and possibly led to the inhibition of testicular antioxidative ability since SOD, which clears the ROS superoxide radicals, is considered the first line of cell defense against oxidative stress damage (Afonso et al., 2007). The significant decrease of SOD activity in the 0.2- and 0.5-mg-dosed groups further confirmed the reduction of SOD protein and inhibition of its antioxidative capabilities following PFDoA exposure. Thus, the ability of the testicular cells to clear away superoxide radicals may be weakened by PFDoA exposure. Fth1 sequesters iron that is able to react with ROS in order to avoid the formation of highly toxic species (Levi and Arosio, 2004). Therefore, decreased Fth1 protein in this study may augment the testicular oxidative stress response in PFDoA-treated rats. DDT, another antioxidant, has been shown to protect cells from the damage of oxidative stress (Hiyoshi et al., 2009). Reduced protein levels of DDT in this study may also contribute to the inhibition of antioxidative capability in testis. The decrease of Glod4 in animals receiving the highest PFDoA dose implies a compromised ability to detoxify the peroxidative products, which are usually produced during an oxidative stress response, thereby possibly enhancing the toxicity of oxidative stress (Thornalley, 2003). Unexpectedly, we also observed a marked increase in the protein levels of IDO, an antioxidant scavenger (Christen et al., 1990), in testes from rats in the 0.02- and 0.5-mg-dosed groups, suggesting that PFDoA causes complex effects on the testicular antioxidative system in rats. Increased LPO levels in testis following PFDoA exposure, however, indicated that oxidative damage had occurred since LPO elevation has been served as an important indicator of oxidative stress (Orrenius et al., 2007; Parthasarathy et al., 2008). Thus, the increase in IDO may not be sufficient to prevent this deleterious response. Since oxidative stress may disrupt testicular progesterone and testosterone synthesis (Kostic et al., 2000), oxidative damage may be another reasonable explanation for the decreased serum progesterone and testosterone levels (Shi et al., 2009) in the 0.2- and 0.5-mg-dosed groups. Collectively, we speculate that superfluous ROS originating from decreased antioxidative ability and abnormal mitochondrial respiration in testis after PFDoA exposure leads to increased LPO levels and reduced serum pregnenolone levels.

Six differential proteins that function in sperm activity were altered by PFDoA exposure. AKAP4, a specific sperm flagellum protein, is reported to stimulate sperm motility by recruiting PKA to the fibrous sheath and facilitating local phosphorylation (Eddy et al., 2003; Miki et al., 2002). The decreased AKAP4 protein following PFDoA exposure suggests that this chemical may disrupt PKA-induced phosphorylation by affecting the interactions between AKAP4 and PKA, leading to alterations in sperm motility. Acid phosphatase 1, soluble isoform A (PP1A) may disrupt AKAP4 function by restricting the interaction between AKAP4 and PKA (Chakrabarti et al., 2007). Thus, increased PP1A may further inhibit the ability of AKAP4 to mobilize PKA and disrupt AKAP4 function. PFDoA may lead to reduced fertility via inhibition of AKAP4 function. Indeed, AKAP4 knockout mice are infertile due to the lack of sperm motility (Miki et al., 2002). In addition, the decreased protein levels of YWHA and DJ-1 in the 0.5 mg PFDoA/kg/day treatment group further suggests that high PFDoA concentration may inhibit sperm motility since these two proteins play important roles in supporting both sperm motility and overall male fertility (Huang et al., 2004; Okada et al., 2002). PEBP1 specifically binds phospholipids and acts as a decapacitation factor in sperm maturation,

thus playing an important role in maintaining normal fertilizing ability (Nixon et al., 2006). The significant decline of PEBP1 levels following PFDoA exposure implies that PFDoA may disrupt fertility possibly through inhibition of sperm decapacitation. In general, capacitation is thought to be highly correlated with protein tyrosine phosphorylation induced by protein tyrosine kinase (PTK), such as CSTK, and the presence of albumin (Visconti et al., 1995). The observed decreased levels of CSTK and albumin indicate that PFDoA may exhibit negative effects on sperm capacitation. This result also suggests that PFDoA may impact fertility, since capacitation is a prerequisite for fertilization. Collectively, our results suggest that PFDoA yields adverse effects on male fertility possibly through disrupting sperm activity, although sperm viability parameters were not analyzed in this study.

Some cytoskeleton-associated proteins, including Rho, GDP dissociation inhibitor beta (RhoGDI-β), keratin 10, stathmin 1, and glia maturation factor-gamma (GMFG) were also altered by chronic PFDoA exposure. These proteins play important roles in supporting testicular structure and function including spermatogenesis and testicular cell morphology through cytoskeleton reorganization (Guillaume et al., 2001; Kierszenbaum and Tres, 2004; Lui et al., 2003; Tsuiki et al., 2000). Our observed changes in the expression of these proteins following chronic PFDoA exposure suggest that cytoskeleton reorganization may participate in the PFDoA-induced toxic effects on testicular function.

In addition, proteins associated with protein quality control such as ubiquitin-conjugating enzyme E2N and constitutive photomorphogenic homolog subunit 8, both of which mediate intracellular proteolysis (Glickman and Ciechanover, 2002), were also identified as altered following PFDoA exposure. Furthermore, the expression levels of several proteins involved in intracellular signal transduction were also changed by chronic PFDoA exposure. Since protein quality control system and the signaling molecules are easily affected by various biological processes, the changes of signaling transduction may result from the cooperation of multiple pathways including dysfunction of mitochondrial respiration and oxidative stress in the testes of PFDoA-treated rats. Above results also suggest that alteration in proteolysis pathway and signal transduction may play a role in testicular toxicity of PFDoA.

In order to explore the relationship between transcription and translation, the transcriptional levels of the eleven proteins that were significantly altered by PFDoA exposure were examined. The mRNA levels of most of the genes did not exhibit changes similar to those observed for their protein levels in testis. SOD and H-ATPase subunit d, however, displayed a generally consistent downregulated trend in both mRNA and protein levels, which were also in accordance with SOD and H-ATPase activity alterations. These results indicated that these two enzymes are more sensitive to PFDoA than the other proteins analyzed in this study. These results also imply that mitochondrial disruption and oxidative stress may play important roles in the testicular toxicity that results from PFDoA exposure. As was shown in other recent studies performing parallel proteomic/gene expression studies on the effects of dioxin (Pastorelli et al., 2006) on rats or interferons on the SHK-1 cell line from Atlantic salmon (Martin et al., 2007), the relationship between mRNA transcription and the abundance of protein is not always a direct one as many regulatory mechanisms can affect these processes. Our results suggest that the gene expression response to PFDoA involves diverse regulatory mechanisms from transcription of mRNA to the formation of functional proteins.

In conclusion, this proteomic analysis provides an overview of proteome changes in the testes of rats chronically exposed to PFDoA. Our results demonstrate that testicular toxicity of PFDoA may be associated with the alteration of expression of proteins involved in mitochondrial respiratory chain, oxidative stress, sperm activity, cytoskeleton, and intracellular signaling transduction in rat testes. The mitochondrial disruption and oxidative stress play important roles in progesterone and testosterone inhibition of PFDoA. SOD and H-ATPase subunit d may be sensitive molecular for PFDoA toxic action in testis. These findings provide new insight into the molecular mechanism of PFDoA action in testis. The role of the proteins identified in this study will be the main focus of future studies on the mechanisms of PFDoA toxicity.

Conflict of interest statement

The author declares that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxlet.2009.10.016.

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