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Lipid homeostasis and oxidative stress in the liver of male rats exposed to perfluorododecanoic acid

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Abstract

Perfluorododecanoic acid (PFDoA), a perfluorinated carboxylic acid (PFCA) with twelve carbon atoms, has broad industrial applications and is widely distributed in both wildlife and the environment. Unlike other PFCAs with short carbon chain, however, limited studies have been performed to date on the toxic effects of PFDoA on animals. To determine the hepatotoxicity of PFDoA, male rats were orally dosed by gavage for 14 days with 0, 1, 5, or 10 mg PFDoA/kg/day. Absolute liver weights were diminished, but the relative liver weight was significantly increased in the 5 and 10 mg PFDoA/kg/day groups. Meanwhile, serum triglyceride (TG) concentrations were decreased significantly in rats dosed with 1 and 5 mg PFDoA/kg/day, while the liver lipid accumulation was observed in ultrastructure. The expression of peroxisome proliferator-activated receptor (PPAR)-α and its target genes, and to a lesser extent PPARγ, was induced by PFDoA. No significant changes in the expression of liver X receptor α (LXRα) or its target genes CYP7A1 and acetyl-CoA carboxylase 1 (ACC1) were noted, although the mRNA levels of several genes involved in lipogenesis and lipid transport were changed significantly in the certain of the experimental groups. In addition, superoxide dismutase (SOD) and catalase (CAT) activities were activated significantly in the 1 mg PFDoA/kg/day group and inhibited significantly with a concomitant increase of lipid peroxidation (LPO) levels in the 5 and 10 mg PFDoA/kg/day groups. Our results demonstrate that PFDoA exerts notable hepatotoxicity in male rats and that PPAR and its target genes, SOD and CAT activity, and LPO levels exhibited sensitivity to the toxicity of PFDoA.

Keywords: PFDoA; Male rats; Hepatotoxicity; PPAR; LXRα; Oxidative stress

Introduction

Perfluorinated carboxylic acids (PFCAs, $C_nF_{2n+1}COOH$) have been commercially produced for more than 50 years. Due to their oleophobic, hydrophobic nature, and chemical stability, PFCAs are widely used in industry and consumer products, such as surfactants, lubricants, adhesives, paints, fire fighting foams, and cosmetics (Giesy and Kannan, 2001). The strong carbon–fluorine bond in PFCAs contributes to its utility but also results in a high degree of environmental persistence and bioaccumulation. PFCAs are distributed globally in both the environment and wildlife (Giesy and Kannan, 2001; Kannan et al., 2002a,b; Moody et al., 2002), even in remote areas such as the Arctic (Martin et al., 2004; Smithwick et al., 2006).

Perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) are the predominant perfluorinated pollutants and, thus, have received worldwide attention. Some studies have shown that PFOA and PFOS exert reproductive and developmental toxicities, decrease body weight and serum lipid levels, and induce adenoma in hepatocytes and testicular Leydig cells in laboratory animals (Biegel et al., 2001; Lau et al., in press). In addition, the liver is the primary organ for bioaccumulation and toxicity in rodents (Hundley et al., 2006; Seacat et al., 2003). Most of the effects of these PFCAs on the liver, including increased liver-to-body weight ratios, hepatocellular hypertrophy, vacuolation (Seacat et al., 2002), peroxisome proliferation, and changes in lipid metabolism (Kenney et al., 2004), were

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initiated by the activation of peroxisome proliferator-activated receptors (PPARs).

PPARs are members of the nuclear receptor superfamily that are central regulators of lipid homeostasis (Ferre, 2004; Vanden Heuvel et al., 2006). In response to ligand activation, all PPAR isoforms (PPARα, PPARβ(δ), and PPARγ) bind to peroxisome proliferator response elements (PPREs) as heterodimers with the retinoid X receptor (RXR). This binding modulates expression of target genes involved in lipid metabolism and synthesis, such as acyl CoA oxidase (ACOX), carnitine palmitoyltransferase 1 (CPT1), CYP4A1, and mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) (Shearer and Hoekstra, 2003). Recently, several studies have suggested that PFCAs activate PPAR isoforms and their target genes (Maloney and Waxman, 1999; Takacs and Abbott, 2006; Vanden Heuvel et al., 2006). In addition, the activity or expression of several hepatic enzymes involved in fatty acid metabolism and cholesterol synthesis was altered following low-dose PFCA treatments (Guruge et al., 2006; Haughom and Spydevold, 1992; Intrasuksri et al., 1998). Furthermore, the relationships between increased lipid droplet formation and enhanced fatty acid oxidation in the liver and between increased liver lipids and reduced serum lipids in animals exposed to PFCAs are unclear although several researchers have suggested that these effects were correlated with lipid transfer and synthesis (Haughom and Spydevold, 1992; Kudo et al., 1999).

The liver X receptors (LXRα and LXRβ) are ligand-regulated nuclear receptors that heterodimerize with the RXR and play an important role in hepatic bile acid and fatty acid synthesis (Willy et al., 1995; Schultz et al., 2000). In human embryonic kidney 293 cells, unsaturated fatty acid binds to LXRα and antagonizes oxysterol activation of LXRα but not LXRβ (Ou et al., 2001; Pawar et al., 2002). These fatty acids, thus, interfere with LXR/RXR binding to DNA regulatory elements (Yoshikawa et al., 2002). Nevertheless, Pawar et al. (2003) reported that LXRα is not a target for unsaturated fatty acid regulation in primary rat hepatocytes or in the liver, although some LXR-regulated gene products such as ATP-binding cassette subfamily A member 1 (ABCA1) and sterol regulatory element binding protein 1c (SREBP-1c) were down-regulated by fatty acids. The ability of PFCAs that have a similar structure to fatty acids to alter the mRNA expression of LXRs and target PPARs is not clear (Vanden Heuvel et al., 2006). Additionally, another SREBP isoform, SREBP-2, is a key regulator of cholesterol homeostasis and acts as a transcription factor for HMG-CoA reductase (HMGR) and low density lipoprotein receptor (LDLR) (Miserez et al., 1997). PFOA and PFOS have been reported to change the expression or the activity of HMGR, but the results were inconsistent (Haughom and Spydevold, 1992; Martin et al., 2007). In addition, reactive oxygen species (ROS) such as H_2O_2 and O_2^- may be produced during fatty acid oxidation and can damage the liver cells if allowed to persist. PFOS and PFOA induce ROS production and a corresponding antioxidative response in fish hepatocytes and the human hepatoma cell line HepG2 *in vitro*, resulting in oxidative damage such as lipid peroxidation and DNA damage (Liu et al., 2007; Panaretakis et al., 2001). The effects of these compounds, however, are not clearly defined *in vivo*.

Perfluorododecanoic acid (PFDoA) is a PFCA that contains twelve carbon atoms. Several studies with PFCAs of seven to ten carbon atoms indicated that a longer carbon chain length was eliminated more slowly, thus, was more bioaccumulative (Kudo et al., 2001, 2006; Ohmori et al., 2003) and was shown to be more toxic than shorter carbon length (Olson and Andersen, 1983; Kudo et al., 2006). Moreover, the PFOA elimination rates are dramatically different in different species [female rat (hrs) ≫ male rat (days) > mouse > monkey (weeks) > human (years)] (Lau et al., in press). Taken together, these characteristics indicate that PFDoA is likely to have different toxicity to human and animal populations from PFDA, PFOA, or PFOS. Limited studies, however, have been performed to date on the toxic effects of PFDoA on animals (Shi et al., 2007).

The purpose of the current study was to determine whether PFDoA exposure has similar effects in the male rat liver as other PFCAs that have shorter carbon lengths. The body weight, relative liver weight, and serum and liver lipid levels of male rats that had been exposed to PFDoA by oral gavage were measured. Changes in liver ultrastructure were observed as an additional marker of PFDoA exposure. Furthermore, two important nuclear receptors (PPARs, LXRα) and some genes responsible for lipid homeostasis were studied in order to clarify the effect of PFDoA on lipid metabolism and transport. Finally, concomitant with the study of the oxidative stress induced by PFDoA in rat livers, superoxide dismutase (SOD) activity, catalase (CAT) activity, and lipid peroxidation (LPO) levels were determined to elucidate the mechanism of PFDoA toxicity in the liver.

Materials and methods

Animals. Male Sprague–Dawley rats (230–240 g) were obtained from Weitong Lihua Experimentary Animal Central (Beijing, China). Animals were housed two per cage and maintained in a mass air displacement room with a 12-hour light–dark cycle at 20 to 26 °C with a relative humidity of 30 to 70%. Animals had access to food and water ad libitum. After 1 week of adaptation, the rats were separated into four groups of ten rats each according to mean body weight.

Chemicals and treatments. PFDoA (CAS No.307-55-1, >99% purity) was purchased from Sigma Aldrich, USA. All other chemicals and reagents were analytical grade. PFDoA was suspended in 0.5% Tween-20 (Beijing Chemical Reagent Co. Beijing, China) daily and given orally via gavage to rats for 2 weeks at doses of 1, 5, and 10 mg/kg body weight/day. Control rats were treated similarly but given vehicle only. The doses chosen for this study were based on a preliminary trial in which all rats died during a 14-day treatment with 20 mg PFDoA/kg body weight/day. The PFDoA suspension and the control Tween-20 solution were administered in a volume of 6 ml/kg body weight due to the accuracy of delivery of this volume. After 14 days of treatment, the rats were weighed, and six rats from each group were euthanized by decapitation. The remaining four rats were continually received PFDoA or vehicle for 28 days and sampled for proteomic analysis. Blood was collected and centrifuged at 2000×*g* at 4 °C for 15 min. Serum was stored at −20 °C until analysis. Livers were immediately isolated and weighed. One part of the liver was fixed in 2.5% gluteraldehyde, and another part was immediately frozen in liquid nitrogen and stored at −80 °C until used for RNA isolation.

Serum and liver lipids. Liver lipids were extracted with a mixture of chloroform/methanol (2:1 v/v). Serum TG and liver TG and cholesterol were analyzed with commercial kits according to the manufacturer's recommendations (Biosino Bio-technology and Science Inc., Beijing, China).

Table 1
Sequences of primers used for real-time RT-PCR amplification

Target gene	GenBank accession no. [a]	5′→3′ primer sequences [b]	Product length (bp)	Tm (°C)
β-actin	NM_031144	FW: TCGTGCGTGACATTAAAGAG	134	56.0
		RW: ATTGCCGATAGTGATGACCT		50.0
PPARα	NM_013196	FW: TGAAAGATTCGGAAACTGC	110	56.0
		RW: TCCTGCGAGTATGACCC		
PPARγ	NM_013124	FW: GTTGATTTCTCCAGCATTTC	119	50.0
		RW: TTGATCGCACTTTGGTATT		
ACOX	J02752	FW: GATAATTGGCACCTACGC	98	56.0
		RW: TGAACTCTTGGGTCTTGG		
CPT1	NM_001004085	FW: GTTCTTCGAGCTGGATGTG	137	56.0
		RW: GGTGGTTGGAGGTTAGGAT		
CYP4A1	M14972.1	FW: CCACCACAACCCGAAGG	98	56.0
		RW: TGAGAAGGGCAGGAATGA		
ACC1	NM_022193	FW: TGAAGGGCTACCTCTAATG	182	50.0
		RW: TCACAACCCAAGAACCAC		
APOB	NM_019287	FW: CGCTGAAATGAAACTCG	350	50.0
		RW: CACCCTGAACCGTAGC		
LXRα	NM_031627	FW: GCTCAAGCCACATCGG	101	50.0
		RW: GCAGCCACCAACTTCTCA		
CYP7A1	NM_012942	FW: CTGGCTGAGGGATTGAA	134	50.0
		RW: ATAGCGAGGTGCGTCTT		
SREBP-1c	XM_213329	FW: TGGAGCGAGCATTGAA	117	50.0
		RW: CGACAGCGTCAGAACAG		
SCD-1	NM_139192	FW: CTCATCATTGCCAACACCA	149	50.0
		RW: ACAAGCAGCCAACCCAC		
HMGR	NM_013134	FW: GAGAATAAACCAAACCCAG	94	56.0
		RW: ATCAGCTATCCAGCGACT		
ABCA1	NM_178095	FW: CCCGGCGGAGTAGAAAGG	67	56.0
		RW: AGGGCGATGCAAACAAAGAC		
LDLR	NM_175762	FW: GGCTATGAGTGCCTATGTC	209	56.0
		RW: GTGAAGAGCAGAAACCCTA		

[a] GenBank accession number (http://www.nvbi.nlm.nih.gov) used to design the primers.
[b] FW: forward primer; RW: reverse primer.

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

Real-time RT-PCR. Total RNA was isolated from the liver samples using the 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. One microgram of total RNA was subjected to reverse transcription using an oligo-$(dT)_{15}$ primer (Promega, USA) and M-MuLV reverse transcriptase (New England Biolabs, UK) according to the manufacturers' instructions. Real-time PCR amplification and detection were performed on a Stratagene Mx3000P qPCR system (Stratagene, USA) using SYBR Green technology. The reaction mixture was composed of 12.5 μl of SYBP Premix Ex Taq (Takala, Dalian, China), forward and reverse primers (10 μM, 0.5 μl each), 0.5 μl of ROX reference Dye II, 10 μl of nuclease-free water, and the cDNA sample (1 μl). PCR primers (Table 1) were designed using Primer Premier 5.0 software. The housekeeping gene β-actin was used as an internal control. The differences of efficiencies in amplification between the target genes and β-actin were all less than 5%. The PCR amplification protocol was 95 °C for 15 min followed by 40 cycles of 94 °C for 5 s, 50/56 °C for 15 s, and 72 °C for 10 s. After PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product, which was displayed as a single peak (data not shown). Every sample was analyzed in triplicate. The relative expression ratio (R) of a target gene is expressed for the sample versus the control in comparison to the β-actin gene. R is calculated based on the following equation (Pfaffl, 2001): $R=2^{-\Delta\Delta Ct}$, where C_t represents the cycle at which the fluorescence signal is first significantly different from background and $\Delta\Delta C_t$ is $(C_{t,\ target}-C_{t,\ actin})_{\ treatment}-(C_{t,\ target}-C_{t,\ actin})_{\ control}$.

Biochemical analyses. To determine the antioxidant enzymatic activities and the thiobarbituric acid-reactive substances (TBARS) levels, liver samples were homogenized using a Fluko F8 superfine homogenizer (Fluko, Shanghai, China) in a cold physiological saline solution. The homogenates were then centrifuged at 3000 rpm for 20 min at 4 °C, and the supernatants were analyzed for SOD and CAT activities as well as for TBARs contents using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's

Table 2
Absolute and relative liver weights of rats treated with PFDoA for 14 days

Doses (mg/kg/day)	0	1	5	10
Liver weight (g)	12.00±0.53	12.44±0.43	9.72±0.4*	8.91±0.57*
Relative liver weight (%) [a]	3.85±0.11	4.14±0.11	4.82±0.13*	5.01±0.35*

Data are given as mean±SEM from six rats per group for liver weight and relative liver weight.
[a] Percentage of total body weight (Shi et al., 2007).
* Significant difference from control, $p<0.05$.

Table 3
Concentrations of triglycerides (TG) in the serum and TG and cholesterol in the liver of rats treated with PFDoA for 14 days

Doses (mg/kg/day)	0	1	5	10
Serum (mmol/l)				
TG	0.69±0.08	0.30±0.03	0.34±0.09	1.35±0.64*
Liver (μmol/g)				
TG	43.95±2.15	44.76±3.65	46.29±3.23	55.74±4.00*
Cholesterol	20.48±1.20	18.15±1.57	20.02±1.75	28.43±4.32*

* Significant difference from control, $p<0.05$.

instructions. One unit of SOD activity was defined as the amount of enzyme required to inhibit oxidation by 50% in a 1-ml reaction solution and was expressed as U/mg protein. One unit of CAT activity was defined as the amount of enzyme required to consume 1 μmol H_2O_2 in 1 s and was expressed as U/mg protein. The content of TBARS was expressed as nanomoles of malondialdehyde per mg protein, the method was calibrated with 1,1,3,3-tetraethoxypropane standard solution. Protein content was estimated with a modified Lowry protein assay kit (Applygen Technologies Inc, Beijing, China) using bovine serum albumin as a standard. Each parameter was measured in triplicate.

Statistical analyses. Data were analyzed using SPSS for Windows 13.0 Software (SPSS, Inc., Chicago, IL) and presented as means with standard errors (mean±SE). Differences between the control and the treatment groups were determined using a one-way analysis of variance (ANOVA) followed by the Ducan[a] multiple range test. A p-value of <0.05 was considered statistically significant.

Results

Organ weights

The liver weights of male rats treated for 14 days with PFDoA are shown in Table 2. No significant changes in liver weight or relative liver weight were observed following exposure to 1 mg PFDoA/kg/day compared with controls. For animals exposed to 5 and 10 mg PFDoA/kg/day, the absolute liver weights were significantly reduced by 19% and 26%, respectively ($p<0.05$). However, the relative liver weights were markedly increased in male rats that received 5 and 10 mg PFDoA/kg/day compared to controls ($p<0.05$). The results at 10 mg PFDoA/kg/day are likely confounded by poor health of the animal, even if all rats dosed with 10 mg PFDoA/kg/day were free of clinical signs of toxicity and survived until the end of the study. So the entire results and discussion were focused on the data for the lower doses.

Lipid levels in serum and liver

Rats that received 1 and 5 mg PFDoA/kg/day exhibited slightly reduced serum TG, but no statistical difference was observed ($p>0.05$) (Table 3), while serum TG was increased significantly in 10 mg PFDoA/kg/day groups ($p<0.05$). However, if the data of 10 mg/kg/day groups were excluded in

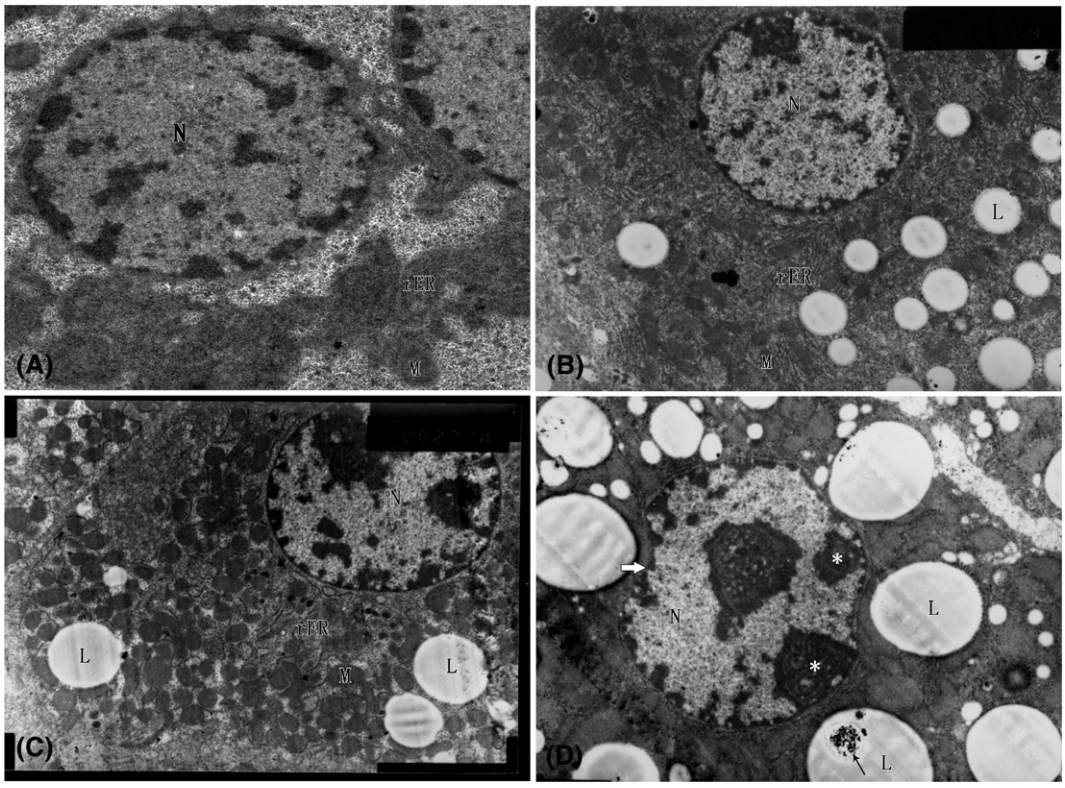

Fig. 1. Rat liver electron microscopy examination following PFDoA treatment for 14 days. (A) Normal control (×6700). (B) 1 mg PFDoA/kg/day group (×5000). Smaller lipid droplets (L) occurred in the cytoplasm. (C) 5 mg PFDoA/kg/day group (×5000). Lipid droplets occurred. Mitochondria (M) proliferated and swelled, and the rough endoplasmic reticulum (rER) was reduced and degranulated in the cytoplasm. (D) 10 mg PFDoA/kg/day group (×4000). Larger lipid droplets (L) and irregular nuclei (empty arrow) with condensed chromatin (*) were observed. The myeloid body occurred in the lipid droplets (arrow).

ANOVA, a significant decrease would be observed in the serum TG levels in 1 and 5 mg/kg/day PFDoA groups ($p<0.05$).

No significant differences in liver lipid levels were observed in the 1 and 5 mg/kg/day groups compared to the controls ($p>0.05$), while liver cholesterol and TG levels were induced significantly in rats receiving 10 mg PFDoA/kg/day ($p<0.05$).

Hepatic histopathology

The rat liver ultramicrostructure was changed after PFDoA treatment compared to control treatment (Fig. 1). Lipid droplet accumulation was observed in the cytoplasm of the hepatocytes of male rats exposed to PFDoA, and the lipid droplets were larger in the higher dose PFDoA groups (Figs. 1B–D). In addition, the rough endoplasmic reticulum (rER) was obviously broken and degranulated in the rats that had received 5 or 10 mg PFDoA/kg/day. At doses of 10 mg PFDoA/kg/day, the nuclear membranes became irregular and were surrounded by large lipid droplets (Fig. 1D).

Gene expression

The effects of PFDoA exposure on the mRNA expression of genes involved in lipid homeostasis in male rats were determined by real-time RT-PCR (Figs. 2–4). Hepatic lipid homeostasis is maintained by balanced lipid formation (lipogenesis), catabolism (β-oxidation), and secretion. The RT-PCR results demonstrated that the mRNA levels of PPARα, a major regulator in hepatic fatty acid β-oxidation, were significantly increased 3.7-fold in the 1 mg PFDoA/kg/day group and 2.8-fold in the 5 mg PFDoA/kg/day group compared to the controls ($p<0.05$) (Fig. 2A). Consistent with the induction of PPARα, the mRNA levels of CPT1, ACOX, and CYP4A1, three important PPARα target genes, were significantly induced in all treatment groups compared to controls

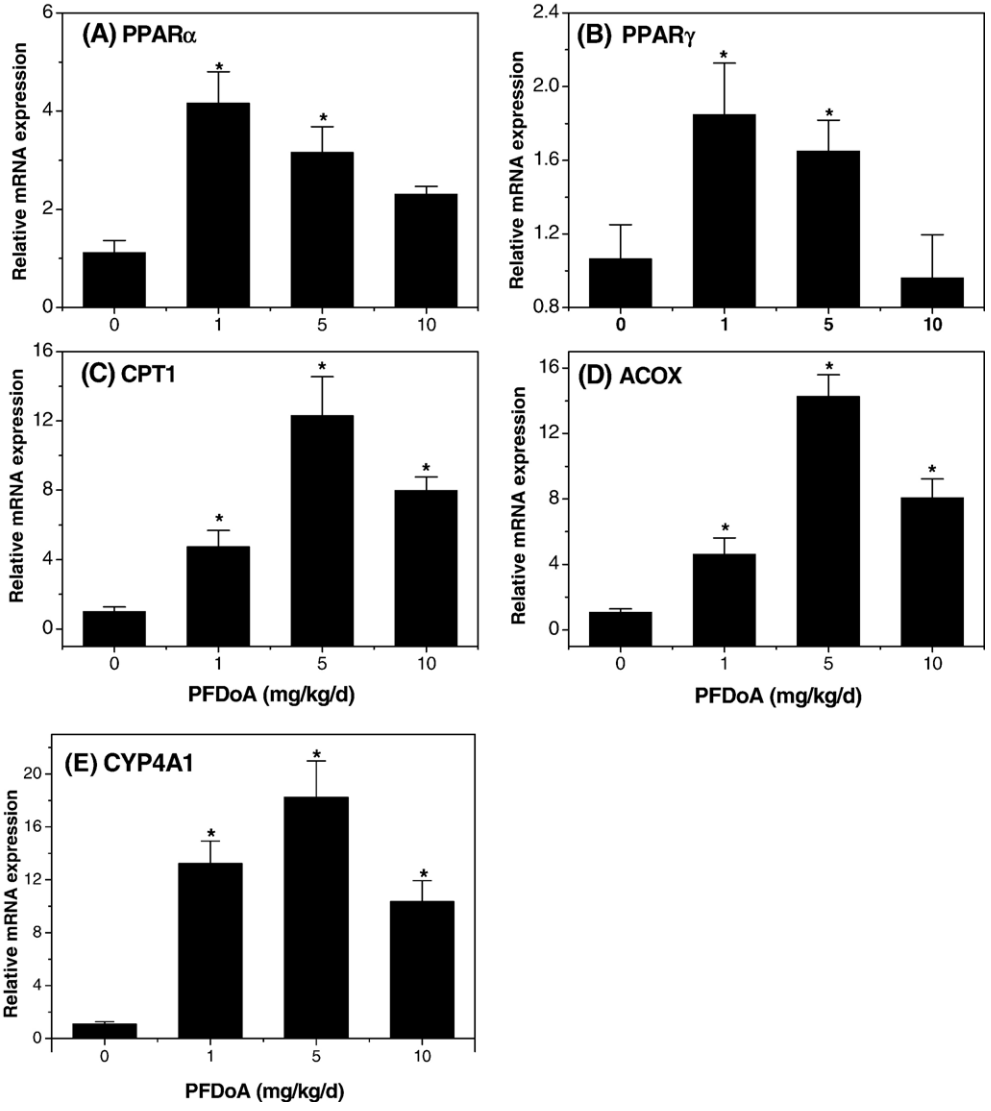

Fig. 2. Real-time quantitative RT-PCR analyses of hepatic mRNA expression levels of (A) PPARα, (B) PPARγ, (C) CPT1, (D) ACOX, and (E) CYP4A1 from control and PFDoA-exposed male rats. Gene expression levels represent the relative mRNA expression compared to controls. Values are presented as the mean ± SEM for six rats per group. Asterisks (*) indicate a statistically significant difference, $p<0.05$.

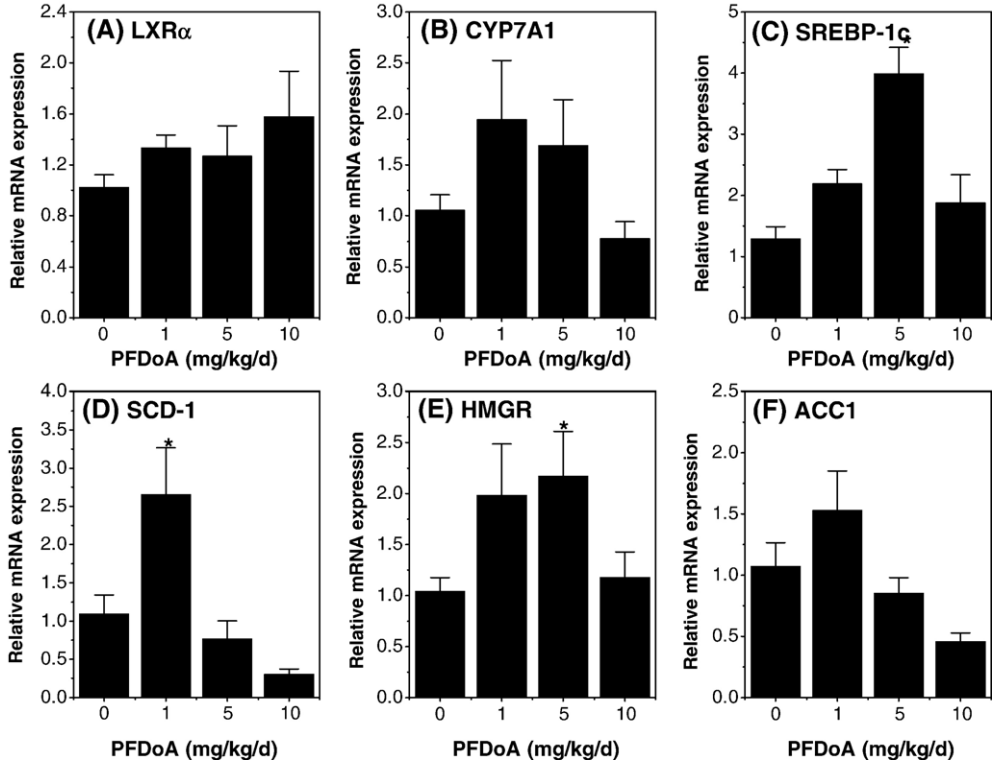

Fig. 3. Real-time quantitative RT-PCR analyses of hepatic mRNA expression levels of (A) LXRα, (B) CYP7A1, (C) SREBP-1c, (D) SCD-1, (E) HMGR, and (F) ACC1 from control and PFDoA-exposed male rats. Gene expression levels represent the relative mRNA expression compared to controls. Values are presented as the mean ± SEM for six rats per group. Asterisks (*) indicate a statistically significant difference, $p<0.05$.

($p<0.05$). In the 5 mg/kg/day group, the expression of CPT1, ACOX and CYP4A1 was increased 12.2-, 13.0-, and 16.8-fold, respectively, compared to the controls (Figs. 2C–E). Similarly to PPARα, the mRNA level of PPARγ, an orphan nuclear receptor known to play a role in lipid storage, was also induced 1.7- and 1.5-fold for the 1 and 5 mg PFDoA/kg/day treatments, respectively ($p<0.05$; Fig. 2B).

Compared to the control group, no significant change occurred in the expression of the nuclear receptor LXRα for 1 and 5 mg PFDoA/kg/day treatments ($p>0.05$; Fig. 3A). Similarly, expression of the LXRα target gene, cholesterol 7α-hydroxylase (CYP7A1), was increased in the 1 and 5 mg/kg/day groups, but the difference was not statistically significant ($p>0.05$; Fig. 3B). SREBP-1c, another regulated gene of LXRα that also plays a major regulatory role in the hepatic lipid biosynthesis, was induced significantly 3.1-fold in the 5 mg PFDoA/kg/day group compared to the control group ($p<0.05$) although only a slight increase in the expression of this gene was observed in the other treatment groups ($p>0.05$; Fig. 3C). Lipogenic enzymes, including HMGR, stearoyl-CoA desaturase 1 (SCD-1), and acetyl-CoA carboxylase 1 (ACC1), are target genes of SREBPs. A 2.4-fold increase in SCD-1 mRNA expression was observed in rats receiving 1 mg PFDoA/kg/day ($p<0.05$; Fig. 3D). HMGR, the rate-limiting enzyme of cholesterol synthesis, had

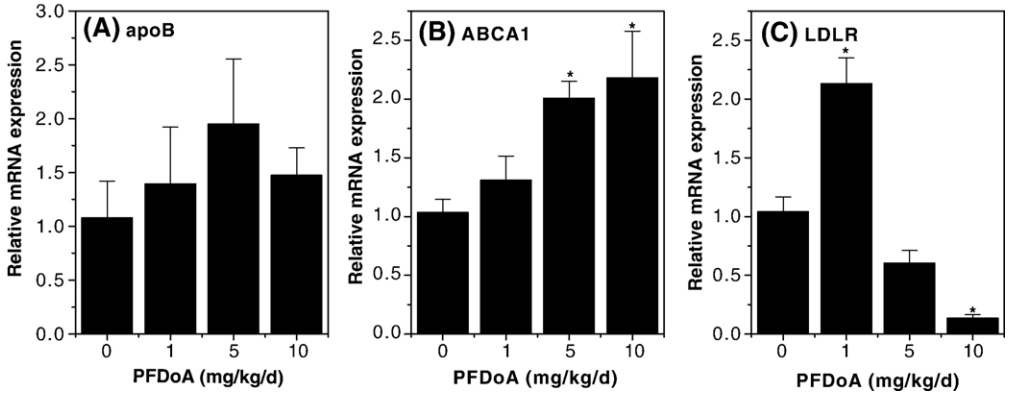

Fig. 4. Real-time quantitative RT-PCR analyses of hepatic mRNA expression levels of (A) ApoB, (B) ABCA1, and (C) LDLR from control and PFDoA-exposed male rats. Gene expression levels represent the relative mRNA expression compared to controls. Values are presented as the mean ± SEM for six rats per group. Asterisks (*) indicate a statistically significant difference, $p<0.05$.

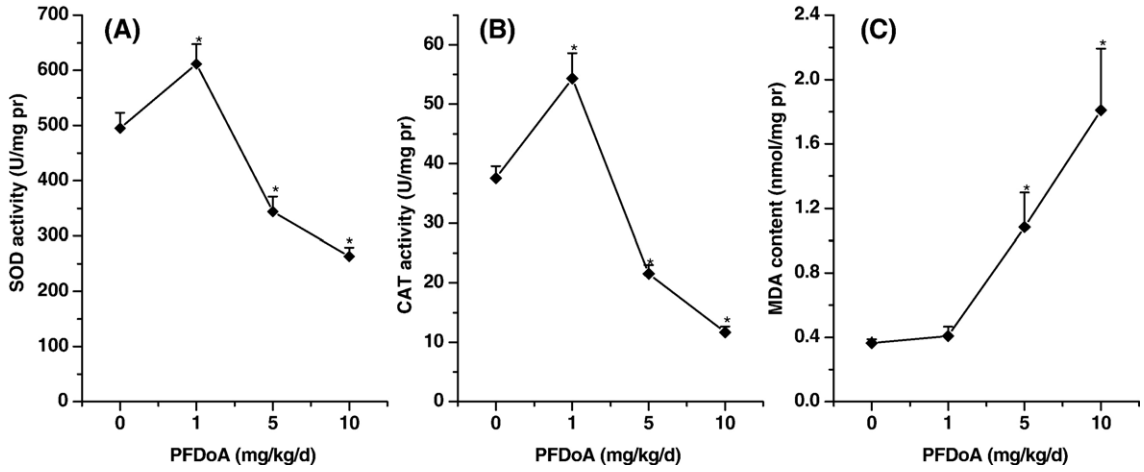

Fig. 5. (A) SOD activity, (B) CAT activity, and (C) LPO levels (measured as TBARS) in the livers of PFDoA-exposed rats. Values are presented as the mean ± SEM for six rats per group. Asterisks (*) indicate a statistically significant difference, $p<0.05$.

the highest mRNA levels (about 2.1-fold of the control) at the dose of 5 mg PFDoA/kg/day ($p<0.05$) with no significant difference between the control and the other treatment groups (Fig. 3E). Although a slight change was noted, no significant differences between the controls and the PFDoA treatment groups were detected for expression of ACC1, which is the rate-limiting enzyme for fatty acid synthesis (Fig. 3F).

Meanwhile, the expression of apoB, which is critical for producing very low density lipoprotein (VLDL) necessary for lipid secretion, exhibited no significant increase in the 5 mg PDDoA/kg/day treatment group (about 1.9-fold of the control; Fig. 4A). On the other hand, ABCA1, which modulates apolipoprotein-mediated efflux of cholesterol, was increased significantly to 1.94-fold in the 5 mg PFDoA/kg/day groups ($p<0.05$; Fig. 4B). In addition, a significant increase in expression (2.05-fold) of LDLR, which is responsible for lipid reverse transport, occurred in the 1 mg PFDoA/kg/day group ($p<0.05$; Fig. 4C).

Activities of antioxidative enzymes and lipid peroxidation

SOD activity significantly increased (23%) in the 1 mg PFDoA/kg/day treatment group compared with the control ($p<0.05$); however, in the 5 mg/kg/day treatment groups, SOD activity exhibited significantly lower levels than the controls (30%; $p<0.05$; Fig. 5A). CAT activity levels were similar to SOD activity. CAT activity was significantly induced in the lower dose group (1 mg/kg/day) and inhibited significantly at the higher 5 mg/kg/day doses ($p<0.05$; Fig. 5B). Levels of TBARS, which are indicative of LPO, were increased significantly by 3-fold compared to the controls for the 5 mg/kg/day groups ($p<0.05$; Fig. 5C). No significant change in TBARS levels was observed in the 1 mg/kg/day group ($p>0.05$).

Discussion

In this study, we investigated the hepatotoxicity of PFDoA in male rats. The relative liver weights were significantly increased in rats receiving 5 mg PFDoA/kg/day. This result is consistent with other studies on the toxicity of PFCAs in rodents and monkeys (Kenney, 1987; Seacat et al., 2002). However, in contrast to other studies on PFOS (Ehresman et al., 2007; Seacat et al., 2002, 2003) and PFOA (Perkins, 1992), absolute liver weights were significantly decreased in rats dosed with 5 mg PFDoA/kg/day, which may be associated with the notable decreases in body weight reported in our previous study (Shi et al., 2007).

The serum TG increased significantly in 10 mg PFDoA/kg/day as well as the serum cholesterol reported by Shi et al. (2007). It was inconsistent with previous studies on PFOA and PFOS which showed that those chemicals cause decreases in serum TG and cholesterol (Haughom and Spydevold, 1992; Kudo et al., 1999; Seacat et al., 2002, 2003). However, the data excluded from the highest dose of 10 mg PFDoA/kg/day were again analyzed, we found that serum TG concentrations were decreased significantly in 1 and 5 mg PFDoA/kg/day groups, while total serum cholesterol concentrations were decreased significantly in rats dosed with 1 mg PFDoA/kg/day. Hence, combined significant body weight loss with biochemical endpoints in the group of 10 mg/kg of PFDoA, response in the highest PFDoA concentration in current study may reflect systemic toxicity.

Hepatic lipid homeostasis is tightly maintained by a balance between lipid formation (lipogenesis), catabolism (β-oxidation), and secretion. PPARα regulates target genes, such as CPT1, ACOX, and CYP4A1. Several studies have shown a significant activation of PPARα by PFCAs in Cos-1 and 3T3-L1 cells using various binding and reporter constructs in cell-based assays (Maloney and Waxman, 1999; Shipley et al., 2004; Takacs and Abbott, 2006; Vanden Heuvel et al., 2006). These published findings are consistent with our results in laboratory rats. Maximal activation of PPARα by PFDoA occurred on Day 14 in the 1 mg PFDoA/kg/day group. Meanwhile, the expression of CPT1, ACOX, and CYP4A1, enzymes that catalyze mitochondrial and peroxisomal fatty acid β-oxidation and unsaturation fatty acid ω-oxidation, respectively, was induced for 14 days in all treatment groups and peaked in rats receiving 5 mg PFDoA/kg/day. The results are in agreement with previous studies that demonstrate the toxicity of PFCAs by determining the activities of ACOX (Ehresman et al., 2007; Intrasuksri et al., 1998) and

peroxisomal β-oxidation (Kudo et al., 2000, 2006; Loveless et al., 2006). Thus, PFDoA induces fatty acid oxidation in the liver of male rats similar to other PFCAs with short carbon chain. PPARγ is a key regulator of lipid storage, adipocyte differentiation, and inflammation control (Berger and Moller, 2002; Hihi et al., 2002). Normally, this factor is expressed at very low levels in the liver; however, expression of PPARγ is greatly increased in animal models with fatty livers (Gavrilova et al., 2003). In the present study, PPARγ mRNA was induced significantly by PFDoA treatment at doses of 1 and 5 mg/kg/day similar to PPARα. These results are consistent with published reports on the effects of PFOA (Elcombe and Wolf, 2004; Vanden Heuvel et al., 2006) but inconsistent with two other reports (Maloney and Waxman, 1999; Takacs and Abbott, 2006), which showed no significant activation of PPARγ by PFOA *in vitro*. The discrepancies may stem from different experimental conditions.

Peroxisome proliferation has been demonstrated to be induced by PFOS in rats and mice (Guruge et al., 2006; Shipley et al., 2004), however, no obvious induction of peroxisome proliferation following PFDoA treatment was observed by electron microscopy in this study, which similar to several long-term repeat-dose studies (Seacat et al., 2002, 2003; Shipley et al., 2004). An equivocal (<2-fold) increase in hepatic palmitoyl CoA activity was observed at 4 weeks in male rats given PFOS (20 ppm in the diets) while no change was observed after 14 weeks despite evidence for increased hepatocytic hypertrophy and vacuolation (Seacat et al., 2003). In *in vitro* studies, Shipley et al. (2004) also found a 3.4-fold increase in ACOX following 50 μM PFOS treatment but no notable effect following 75 μM PFOS treatment. Together, these results suggest a mechanism for downregulation of the hepatic peroxisome proliferation response to PFCAs with high-dose or long-term treatment. Peroxisomes have been demonstrated to generate from domains in the ER (Hoepfner et al., 2005; Tabak et al., 2003). Thus, the break and degranulation of rER caused by PFDoA may also have some influence.

In our study, LXRα mRNA levels were not increased by any dose of PFDoA. This finding was consistent with the report by Vanden Heuvel et al. (2006) in which LXR was unresponsive to PFOA in all species examined. CYP7A1, a target of LXRα and the rate-limiting enzyme of the classic bile acid synthesis pathway (Russell, 1999), was not activated by PFDoA. Thus, PFDoA appeared to have no effect on the conversion of cholesterol to bile acid in the livers of male rats in this experiment by influencing the CYP7A1 expression. Interestingly, SERBP-1c, which is another target gene of LXRα and regulates the expression of many genes that promote fatty acid and TG synthesis, including SCD-1, ACC, and FAS (Shimano et al., 1999), was significantly induced about 4-fold compared to the control in the 5 mg PFDoA/kg/day group. In addition, the expression of SCD-1, the rate-limiting enzyme in the biosynthesis of mono-unsaturated fatty acids, was markedly induced to 2.5-fold of the control at doses of 1 mg PFDoA/kg/day. Consistent with our study, previous publications indicated that PFOA and PFDA significantly induce the activity of SCD-1 in male rats (Kawashima et al., 1989; Yamamoto and Kawashima, 1997). Therefore, PFDoA influences lipid synthesis in rats, although ACC1, the key enzyme in fatty acid synthesis, showed no significant alterations following PFDoA treatment.

Furthermore, HMGR, which is the rate-limiting enzyme in cholesterol biosynthesis, exhibited a significant increase in rats receiving 5 mg PFDoA/kg/day. Gene expression profiles revealed that PFOA and PFOS also upregulated HMGR significantly but concomitantly suppressed numerous cholesterol biosynthesis pathway genes (Martin et al., 2007). Haughom and Spydevold (1992), however, found that PFOS and perfluorooctane sulphonic acid (PFOSA) reduced the activity of liver HMGR to less than 50% of controls. Our finding may have reflected only the mRNA levels and not any changes in the enzyme activities, which may be influenced by other factors. In addition, HMGR as well as SCD-1, ACC1, and SERBP-1c may be regulated not only by SERBPs or LXRs but also by other regulators such as PPARγ (Iida et al., 2002; Schadinger et al., 2005). Finally, a negative feedback effect from cholesterol and fatty acids in the liver may affect the expression levels of LXRα and its target genes.

Normally, lipid accumulation in the liver is not infinite, and increased hepatic uptake and biosynthesis of fatty acids are usually compensated for by increased removal of lipids from the liver (Lavoie and Gauthier, 2006). Very low density lipoprotein (VLDL) secretion is one complicated pathway that eliminates fat from the liver. Extracellular non-esterified fatty acids enter hepatocytes from the plasma and are esterified to TGs. Some TG products are recycled to the cytosol while others are channeled into a TG-rich VLDL precursor associated with apoB. Kudo et al. (1999) speculated that the increase in TG in the liver as well as the decrease of TG in the plasma following treatment with PFOA was associated with a decrease in TG secretion from the liver. The mechanism for such alterations is not known. In addition, PFOA interacted strongly with apoB48 and abrogated the association of apoB48 with lipids in the process of intracellular VLDL assembly, thereby inhibiting VLDL secretion (Okochi et al., 1999). In our study, no significant effect of PFDoA on apoB mRNA levels was observed, indicating that PFDoA does not inhibit VLDL secretion by reducing apoB mRNA expression.

Moreover, ABCA1, which is another target gene of LXRα and modulates apolipoprotein-mediated efflux of cholesterol, was upregulated significantly by PFDoA in 5 mg/kg/day groups. The increase of lipid in the liver concomitant with the increased PFDoA concentration may contribute to the increased efflux of cholesterol from the liver to plasma. PFDoA also induced LDLR expression at the lowest dose, suggesting that the adverse transport of cholesterol may be increased at lower PFDoA doses.

PFOA induced ROS such as H_2O_2 and O_2^- in the human hepatoma HepG2 cells (Panaretakis et al., 2001) and induced SOD and CAT activities in primary cultured hepatocytes from freshwater tilapia (Liu et al., 2007). The activities of SOD and CAT, which are important scavengers of ROS, were significantly increased in rats treated with 1 mg PFDoA/kg/day. This finding suggests that low concentrations of PFDoA may induce ROS production in the livers of male rats perhaps by enhancing fatty acid β-oxidation. The activities of SOD and CAT were, however, inhibited significantly at higher doses of PFDoA, corresponding to significant increases in TBARS levels. This

phenomenon was demonstrated in a previous study on PFOA and PFOS (Liu et al., 2007). The diminished enzyme activities may result, in part, from a delay in protein synthesis as we detected a decrease in rER in this study or from direct inhibition due to the production of superoxide radicals in excess (Kono and Fridovich, 1982).

In summary, based on the selected endpoints, we found that the response observed in low doses of PFDoA (1 and 5 mg/kg/day) groups could better reflect the mode of action of PFDoA than that in 10 mg/kg/day groups. Therefore, low doses of PFDoA exhibited the trend to decrease the serum TG and cholesterol in this study, similar to those seen in other PFCAs. Meanwhile, PFDoA induced the expression of PPARα, PPARγ to a lesser extent, and their target genes to enhance fatty acid β-oxidation. LXRα was not influenced significantly by PFDoA exposure experiment, as well as its target genes CYP7A and ACC1. PFDoA, however, did induce the expression of HMGR, SERBP-1c, and SCD-1, all of which play a role in lipogenesis. Furthermore, PFDoA influenced ABCA1 and LDLR expression; however, expression of these genes was not consistently altered due to possible crosstalk between different nuclear receptors and enzymes. In addition, PFDoA exposure may result in ROS production, which, in turn, may change the SOD and CAT activities as well as the LPO levels. Thus, PPARs and its target genes, SOD, CAT, and LPO, sensitively reflected the effects of PFDoA exposure.

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