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Induction of time-dependent oxidative stress and related transcriptional effects of perfluorododecanoic acid in zebrafish liver

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ABSTRACT

The effects of acute perfluorododecanoic acid (PFDoA) exposure on the induction of oxidative stress and alteration of mitochondrial gene expression were studied in the livers of female zebrafish (*Danio rerio*). Female zebrafish were exposed to PFDoA via a single intraperitoneal injection (0, 20, 40, or 80 μg PFDoA/g body weight) and were then sacrificed 48 h, 96 h, or seven days post-PFDoA administration. PFDoA-treated fish exhibited histopathological liver damage, including swollen hepatocytes, vacuolar degeneration, and nuclei pycnosis. Glutathione (GSH) content and catalase (CAT) activity decreased significantly at 48 h post-injection while superoxide dismutase (SOD) activity was initially decreased at 48 h post-injection but was then elevated by seven days post-injection. The activity of glutathione peroxidase (GPx) increased at 48 h and seven days compared to control fish, although the increased level at seven days post-injection was decreased compared to the level at 48 h post-injection. Lipid peroxidation levels were increased at seven days post-injection, while no apparent induction was observed at 48 h or 96 h post-injection. The mRNA expression of medium-chain fatty acid dehydrogenase (MCAD) was induced, while the transcriptional expression of liver fatty acid binding protein (L-FABP), peroxisome proliferating activating receptor α (PPARα), carnitine palmitoyl-transferase I (CPT-I), uncoupling protein 2 (UCP-2), and Bcl-2 were significantly inhibited. Furthermore, the transcriptional expression of peroxisomal fatty acyl-CoA oxidase (ACOX), very long-chain acyl-CoA dehydrogenase (VLCAD), long-chain acyl-CoA dehydrogenase (LCAD) did not exhibit significant changes following PFDoA treatment. No significant changes were noted in the transcriptional expression of genes involved in mitochondrial respiratory chain and ATP synthesis, including cytochrome *c* oxidase subunit I (COXI), NADH dehydrogenase subunit I (NDI), and ATP synthase F0 subunit 6 (ATPo6). These results demonstrate that turbulence of fatty acid β-oxidation and oxidative stress responses were involved in the PFDoA-induced hepatotoxicity.

1. Introduction

Perfluoroalkyl acids (PFAAs) are a family of perfluorinated chemicals that consist of a carbon backbone with hydrogen replaced by fluorine. The chemical composition renders this class of compounds chemically stable, oleophobic, and hydrophobic and contributes to their wide application in industry and consumer products (Key et al., 1997; Giesy and Kannan, 2001).

Wide commercial use of PFAAs for the past several decades has resulted in their broad distribution in wildlife in terrestrial and aquatic environments (Giesy and Kannan, 2001; Prevedouros et al., 2006; Martin et al., 2004; Smithwick et al., 2006; Yeung et al., 2006; Dai et al., 2006). Meanwhile, their adverse effects have received worldwide attention. Studies in rodents have shown that PFAAs exert reproductive and developmental toxicities, decrease body weight, and interfere with lipid metabolism (Biegel et al., 2001; Lau et al., 2007). The liver was reported to be the primary organ for bioaccumulation, and thus, specifically the toxic effects of PFAAs and the hepatotoxicity were extensively studied (Hundley et al., 2006; Seacat et al., 2003). For example, perfluorooctanesulfonate (PFOS) induced hepatocellular hypertrophy and lipid vacuolation in rats (Seacat et al., 2002). In addition, a toxicogenomic analysis demonstrated that genes involved in the transport and metabolism of fatty acids and lipids, cell communication, adhesion, growth, and apoptosis were significantly altered in perfluorooctanoic acid (PFOA)-treated rats (Guruge et al., 2006).

Several studies have reported the distribution and bioaccumulation of PFAAs in teleosts (Hoff et al., 2005; Olivero-Verbel et al., 2006); however, unlike studies on rodents, studies on the toxic effects in teleosts have focused primarily on PFOA and PFOS.

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Moreover, very few studies have examined the toxic mechanisms of PFAAs in teleosts. An investigation of the hepatotoxicity of PFOS in common carp demonstrated that PFOS interfered with DNA metabolism homeostasis and induced inflammation-independent enzyme leakage although antioxidant levels and peroxisome proliferation were not affected (Hoff et al., 2003). Other *in vivo* studies in fish, including gibel carp (*Carassius auratus gibelio*), carp (*Cyprinus carpio*), eel (*Anguilla anguilla*) fathead minnow (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss)*, showed that PFOS influences hepatocyte membrane function and structure (Hoff et al., 2005), increases hepatic fatty acyl-CoA oxidase activity, and induces oxidative stress (Oakes et al., 2004, 2005). In addition, exposure of PFOS and PFOA for 24 h produced oxidative stress and induced apoptosis *in vitro* (Liu et al., 2007). In a recent study, PFOS induced transcriptional effects on P450 and phase II enzymes in hepatocytes of Atlantic salmon (*Salmo salar*) (Krovel et al., 2008). In our previous study, alterations in gene expression associated with lipid metabolism and transport, hormone action, immune responses, and mitochondrial function were observed in rare minnows (*Gobiocypris rarus*) after PFOA exposure (Wei et al., 2008).

Of the perfluorinated carboxylic acids with a chain length of six to nine carbon atoms, longer chain length species induced greater compound accumulation in the liver and higher resultant toxicity compared with shorter chain length species (Kudo et al., 2006). Moreover, the rate of elimination decreased with increasing carbon chain length and exhibited tremendous species differences (Martin et al., 2003; Olsen et al., 2007). Thus, perfluorododecanoic acid (PFDoA), a compound with 12 carbon atoms, is likely to have different toxic effects than either PFOA or PFOS. Only limited studies of the toxicity of PFDoA have been performed in rats (Shi et al., 2007; Zhang et al., 2008), and to date, no information on PFDoA toxicity in teleosts has been reported.

In order to determine whether PFDoA exerts similar toxic effects on teleosts as other reported PFAAs with shorter carbon lengths, the acute hepatotoxicity of PFDoA on female zebrafish was investigated. The time- and dose-related oxidative stress was evaluated by determination of malondialdehyde (MDA) and glutathione (GSH) content, analysis of the antioxidant activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and observation of histopathlogical changes in the liver. In addition, the transcriptional expression of fatty acid β-oxidation related genes, including liver fatty acid binding protein (L-FABP), peroxisome proliferating activating receptor α (PPARα), carnitine palmitoyl-transferase I (CPT-I), acyl-CoA oxidase (ACOX), very long-chain acyl-CoA dehydrogenase (VLCAD), long-chain acyl-CoA dehydrogenase (LCAD), and medium-chain fatty acid dehydrogenase (MCAD), were examined. Finally, the mRNA levels for mitochondrial inner membrane genes related to reactive oxygen species (ROS) production, respiratory chain, and ATP synthesis were investigated.

2. Materials and methods

2.1. Fish and PFDoA treatment

Adult female zebrafish were purchased from a commercial dealer (Beijing, China) and maintained in aquaria at 28 °C with well-oxygenated water under a photoperiod of 14:10 h light:dark. The aquaria were also set up with continuous water filtration with activated charcoal. Fish were fed daily with a commercial flake food for tropical fish (Tetra, Melle, Germany). The zebrafish were allowed to acclimatize for two weeks before the beginning of the experiment. PFDoA (CAS No.307-55-1, 97% purity) was purchased from Acros Organics (Geel, Belgium) and was dissolved in 30% propylene glycol (Beijing Chemical Reagent Co. Beijing, China). Female zebrafish were randomly divided into four groups and then received a single intraperitoneal injection of PFDoA (10 μL) at doses of either 20, 40, or 80 μg/g body weight. Fish in the control group were treated with the vehicle only. Doses of PFDoA were selected for use in this investigation based on results from a 96-h LD_{50} study, which calculated the LD_{50} value (392 μg/g) with a 95% confidence interval (320–480 μg/g). At the end of the experiment, control and treated fish were anesthetized in ice-cold water and subsequently sacrificed at 48 h, 96 h, or seven days post-injection. Livers were surgically isolated at each designated time point and immediately frozen in liquid nitrogen for stored at −80 °C until analysis. At the seven days time point, hepatic tissues of four individuals per group were quickly dissected and fixed in 10% formalin for histological examination.

2.2. Histopathological analysis

Liver tissues fixed in 10% formalin were processed sequentially in ethanol, xylene, and paraffin. Tissues were then embedded in paraffin wax, sectioned (4–5 μm), and mounted on slides. The sections were subsequently stained with haematoxylin and eosin.

2.3. Biochemical analysis

Liver samples were homogenized using a Fluko F8 superfine homogenizer (Fluko, Shanghai, China) in 10 volumes of cold physiological saline solution (0.85% NaCl). The homogenate was then centrifuged at 2000 × *g* for 10 min at 4 °C to precipitate insoluble materials. The supernatant was used to assay for lipid peroxidation and GSH content as well as for GPx, SOD, and CAT activities using kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In each group, six individual fish were used for biochemical analyses and samples from each fish were performed in triplicate. Lipid peroxidation products (measured as MDA) were quantified by the thiobarbituric acid (TBARS) method, and the MDA concentration was expressed as nanomoles per mg protein. The GSH content was expressed as mg per g protein. GPx activity was defined as the amount of enzyme required to deplete 1 μmol GSH in 1 min, and this activity was expressed as units per mg protein. One unit of SOD activity was defined as the amount of enzyme required to inhibit the oxidation reaction by 50% 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. Protein concentration was assayed using the Bradford protein assay kit (Tiangen, Beijing, China), using bovine serum albumin as a standard.

2.4. Quantitative real-time PCR

Total RNA was extracted from frozen liver tissues using the TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The concentration of total RNA was quantified by the absorbance at 260 nm using a UV1240 spectrophotometer (Shimadzu, Kyoto, Japan). The purity of total RNA was assessed by determining the A_{260}/A_{280} ratio. One microgram of total RNA was reverse-transcribed with an oligo-$(dT)_{15}$ primer and M-MuLV transcriptase as described by the manufacturer (Promega, Madison, USA). The transcriptase was omitted in the negative controls. Quantitative real-time PCR was performed on a Stratagene Mx3000p qPCR system (Stratagene, Cedar Creek, USA). The 25-μL reaction mixture contained 12.5 μL of 2 × SYBR Premix Ex Taq (Takara, Dalian, China), forward and reverse primers (0.1 μM each), 0.5 μL of ROX reference Dye II, nuclease-free water, and 1 μL of cDNA template. The gene-specific primers (Table 1) were designed

Table 1
Sequences of primers used for RT-qPCR amplification

Target gene	Accession no.	Primer sequences (from 5′ to 3′)	Product length (bp)
L-FABP	AF254642	F: AAACCGTCACCAACTCCTTC R: CGACTGTCAGCGTCTCCAC	174
ACOX	NM_001005933	F: ACCCTTCCACCCAAGAGTT R: TTGAGGTTTTACCCAATCCA	84
CPT1	NM_001005940	F: TATGACCGTTCAGACGCAGA R: TACAGGCAGATGTGGCAGAG	241
VLCAD	NM_212611	F: CACAGGTCTTTCCCTATCCC R: AGAGCATCGTTCTTCATCGG	121
LCAD	BC071366	F: TTTACTGGGAGTTTACACACC R: CTTTAGAGCCATAGTGACTGATA	164
MCAD	BC155272	F: CAGAAAGAGTTCCAGGAGGTG R: TGTCCGTTCATTAGACCCAG	137
NDI	AC024175	F: TACAGAGGGGGAATCAGAAC R: TTGGTCGTATCGGAATCGT	253
COXI	AC024175	F: GGATTTGGAAACTGACTTGTG R: AAGAAGAAATGAGGGTGGAAG	105
ATPo6	AC024175	F: TTATCCTCGTTGCCATACTTC R: AGTTGGTTTGTGAATCGTCC	118
Bcl-2	AY695820	F: TCAATAAAGCAGTGGAGGAATC R: TCAAATGAGGGTCTGAACGAG	123
UCP-2	NM_131176	F: TGGCTCAACCCACTGATGTA R: CAATGGTCCGATATGCGTC	102
β-Actin	AF057040	F: ATTGACTCAGGATGCGGAA R: GAGGGCAAAGTGGTAAACG	123

with the Primer Premier 5.0 software. The housekeeping gene β-actin was used as an internal control for normalization. The amplification efficiencies between target genes and β-actin were regulated to differ less than 5%. The amplification protocol was as follows: 95 °C for 10 s followed by 40 cycles of 94 °C for 5 s, 55 °C for 15 s, and 72 °C for 10 s. The relative expression ratio (R) of a target gene was defined as the expression in the treated sample versus the expression in the control sample in comparison to the β-actin gene. R was calculated based on the following equation: $R = 2^{-\Delta\Delta C_t}$, where C_t represents the cycle in which the fluorescence signal is first significant different from background and $\Delta\Delta C_t$ is $(C_{t,target} - C_{t,actin})_{treatment} - (C_{t,target} - C_{t,actin})_{control}$ (Pfaffl, 2001).

2.5. Statistical analyses

In each group, triplicate samples from six different fish were used for biochemical analyses and RT-qPCR. All results were analyzed for significant differences ($p < 0.05$) using a one-way analysis of variance (ANOVA) followed by the Ducan's multiple range test. The data are presented as means with standard errors of the mean (mean ± S.E.).

3. Results

3.1. Hepatic histopathology

Livers of female zebrafish exhibited histopathological changes following acute PFDoA treatment as compared to control (Fig. 1A). Exposure to 20 μg/g PFDoA elicited obvious swelling of hepatic cells (Fig. 1B), while cell borders became obscure and hepatocellular vacuolar degeneration was observed in the 40 μg/g group (Fig. 1C). Hepatic lesions became much more severe in fish exposed to 80 μg PFDoA/g body weight (Fig. 1D). Nuclei enlargement and pycnosis were observed along with concomitant hepatocyte necrosis and cytolysis. The cellular structure was nearly collapsed in livers of fish receiving the highest dose of PFDoA.

3.2. Effect of PFDoA on GSH content

Following PFDoA treatment, a dose-dependent decrease of GSH content was observed (14%, 26%, and 29%) at 48 h (Fig. 2A). After 96 h, fish in the treatment groups of 20 and 80 μg PFDoA/g body weight showed a significant decrease of GSH content compared to control fish (decreased 14% and 12%, respectively; $p < 0.05$). After seven days, rapid depletion of GSH occurred only in the highest dose group (80 μg PFDoA/g). Comparison of GSH content in one dose group at different time points revealed that in the 40 μg PFDoA/g group, fish sacrificed seven days after injection showed a significant elevation of GSH content compared to 48 h (33%, $p < 0.05$), while in the dose group of 80 μg PFDoA/g, fish sacrificed 96 h post-injection exhibited elevated GSH content in comparison to those sacrificed at 48 h (19%, $p < 0.05$).

3.3. Effect of PFDoA on antioxidant enzymes

SOD activity decreased significantly in all PFDoA treatment groups after 48 h, although the differences between the PFDoA treatment groups and the control group became insignificant 96 h post-injection. At seven days post-injection, SOD activity was significantly elevated in the 40 and 80 μg PFDoA/g groups compared to the controls ($p < 0.05$; Fig. 2B). Time-related changes of SOD activity were observed, as fish sacrificed 96 h and seven days after injection showed an obvious elevation in enzyme activity when compared to their respective activities at 48 h post-injection. Specifically, in the 20 μg/g group, SOD activity increased 35% and 19% at 96 h and seven days post-injection, respectively, compared to 48 h. In the 40 μg/g group, SOD activity increased 12% and 25% at 96 h and seven

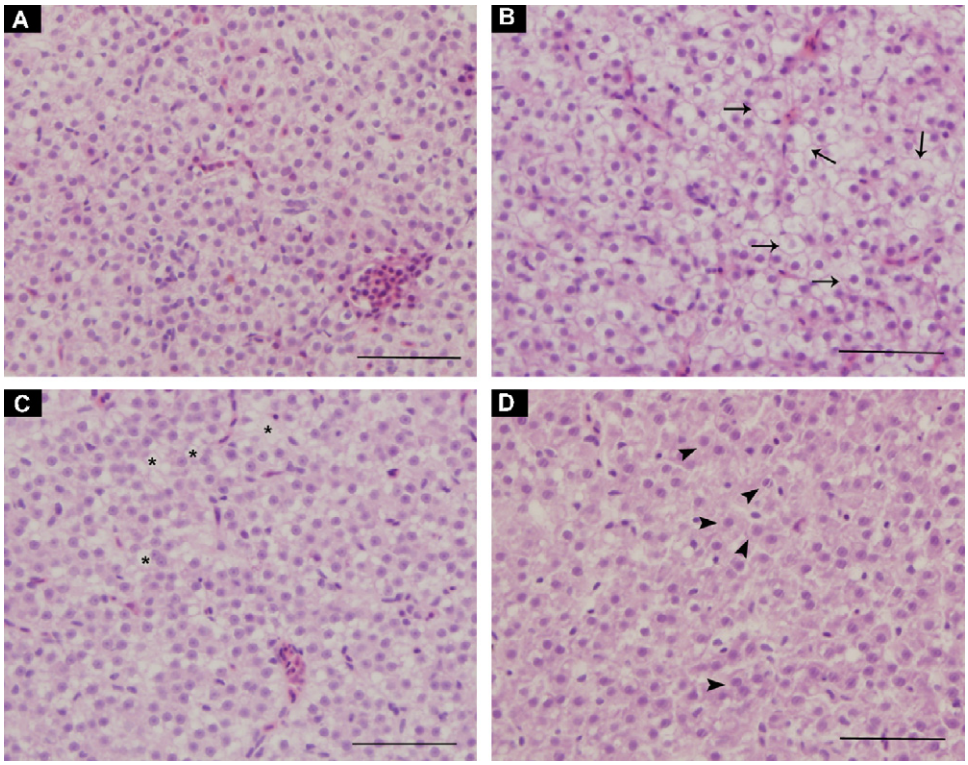

Fig. 1. Liver histopathology of female zebrafish seven days after acute exposure to PFDoA. Photomicrographs of liver sections (4–5 μm) stained with hematoxylin and eosin. (A) Livers from control group. (B) Livers from fish exposed to 20 μg PFDoA/g body weight. A number of hepatocytes were swollen (arrow). (C) Livers from fish exposed to 40 μg PFDoA/g body weight. The hepatocyte borders became obscure, and cell vacuolar degeneration was noted (asterisk). (D) Livers from fish exposed to 80 μg PFDoA/g body weight. Destruction of cellular structures, hepatocyte denaturation, and necrosis were observed. Nuclei became enlarged and pycnotic (arrowhead). Scale bar is equal to 50 μm.

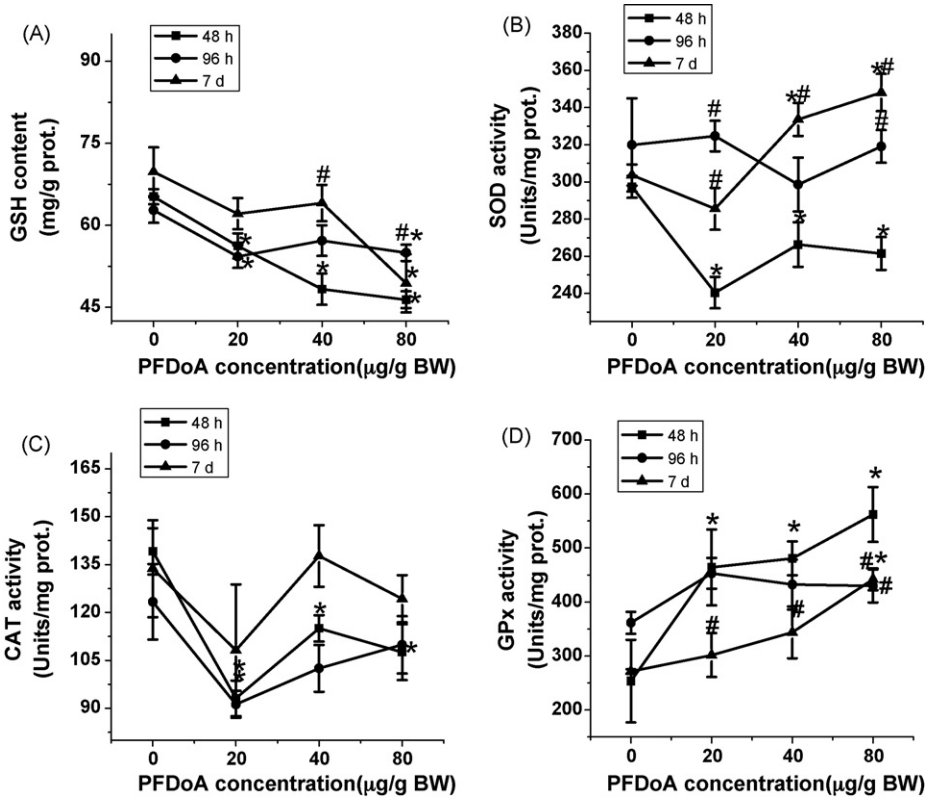

Fig. 2. Time- and dose-dependent alteration of (A) GSH, (B) SOD, (C) CAT, and (D) GPx in livers after acute exposure to PFDoA. Values are presented as the mean ± S.E. from six individual fish. Significance is indicated by * for $p < 0.05$ compared to the control at each time point and by # for $p < 0.05$ compared to 48-h time point in each dose group.

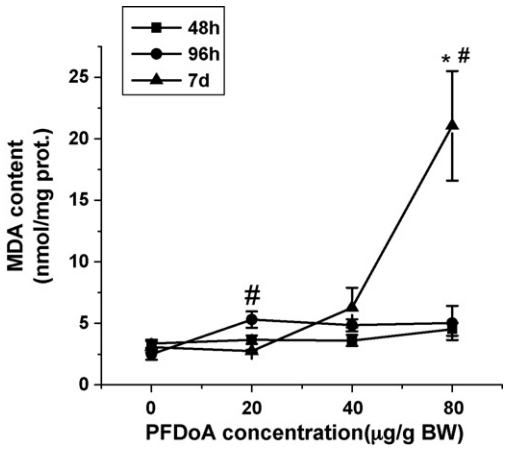

Fig. 3. Time- and dose-dependent induction of lipid peroxidation levels (measured as TBARS) in livers after acute exposure to PFDoA. Values are presented as the mean ± S.E. from six individual fish. Significance is indicated by * for $p < 0.05$ compared to control at each time point and by # for $p < 0.05$ compared to the 48-h time point in each dose group.

days post-injection, respectively. Moreover, in the 80 μg/g group, the increase in SOD activity was more evident with an increase of 22% and 33% at 96 h and seven days post-injection, respectively.

Fish sacrificed 48 h after injection showed significantly decreased CAT activity in all PFDoA treated groups compared to control. However, at 96 h post-injection, only fish exposed to 20 μg/g PFDoA showed a significant 1.4-fold reduction of CAT activity compared to control ($p < 0.05$). As time processed, no significant effects were observed between PFDoA treated fish and the control seven days after injection (Fig. 2C).

GPx activity was induced significantly in a dose-dependent manner 48 h post-injection ($p < 0.05$; Fig. 2D). At 96 h post-injection, no significant difference in GPx activity was observed between PFDoA-treated fish and the control fish. A significant increase of GPx activity (1.6-fold, $p < 0.05$) was observed only in the highest dose group (80 μg PFDoA/g) seven days after injection compared to control. GPx activity was significantly decreased seven days after injection when compared to their corresponding initial activity at 48 h in all the PFDoA-treated groups ($p < 0.05$).

3.4. Effect of PFDoA on lipid peroxidation

Lipid peroxidation levels were not significantly different between the PFDoA-treated groups and their respective controls at 48 and 96 h (Fig. 3); however, seven days after exposure, fish exposed to 80 μg PFDoA/g body weight showed a significant 7-fold increase compared to control fish ($p < 0.05$). In fish exposed to 20 μg PFDoA/g body weight, lipid peroxidation levels were increased 96 h post-injection (1.5-fold, $p < 0.05$) compared to levels at 48 h post-injection; however, this increase was abolished to basal levels seven days post-injection. On the contrary, in the 80 μg/g group, lipid peroxidation levels showed an irreversible increase at later time points compared to 48 h post-injection.

3.5. Effects of PFDoA on gene expression

Effects of PFDoA exposure on mRNA levels of genes involved in fatty acid β-oxidation, mitochondrial respiratory chain, and ATP synthesis were determined by RT-qPCR (Fig. 4). The mRNA level of L-FABP was significantly decreased (2.8-fold, $p < 0.05$) in the 20 μg/g group (Fig. 4A). The mRNA levels of PPARα decreased in a dose-dependent manner with a significant 1.6-, 2.2-, and 2.5-fold decrease in the 20, 40, and 80 μg/g groups, respectively ($p < 0.05$). Consistent with the decreased transcription of PPARα, the mRNA levels of CPT I, the target gene of PPARα, were also significantly decreased with a 2.0-, 1.6-, and 2.2-fold decrease in the 20, 40, and 80 μg/g groups, respectively ($p < 0.05$). On the other hand, the mRNA level of ACOX, another target gene of PPARα that functions as a rate-limiting enzyme in fatty acid peroxisomal β-oxidation, exhibited no significant difference between the control and PFDoA-treated groups. VLCAD, LCAD, and MCAD are a group of chain-length-specific acyl-CoA dehydrogenases that are involved in mitochondrial β-oxidation. No discernable differences in the mRNA expression of VLCAD were observed, while the expression of LCAD was slightly but not significantly elevated ($p > 0.05$). In contrast, the expression of MCAD was induced in the 40 and 80 μg/g groups. In fact, fish in the 40 μg/g group exhibited a significant 2.0-fold induction ($p < 0.05$).

The expression of NDI, COXI, and ATPo6, which are involved in mitochondrial respiratory chain and ATP synthesis, exhibited no significant differences between the control and PFDoA-treatment groups ($p > 0.05$) (Fig. 5A–C). UCP-2 and Bcl-2, which are located in the inner membrane of the mitochondria, play an important role in the regulation of ROS production and antioxidation. UCP-2 expression was inhibited in a dose-dependent manner with a 1.5-, 2.0-, and 2.3-fold decrease in the 20, 40, and 80 μg/g groups, respectively ($p < 0.05$). The expression of Bcl-2 similarly exhibited a 1.8-, 1.6-, and 2.1-fold significant decrease in the 20, 40, and 80 μg/g groups, respectively ($p < 0.05$) (Fig. 5D–E).

4. Discussion

The hepatotoxicity following acute PFDoA exposure in female zebrafish was evaluated for histopathological changes, oxidative stress, and transcriptional effects. PFDoA elicited obvious hepatic lesions, which became more severe with increasing PFDoA concentrations. These observations were very similar to the previously reported changes induced by PFOA exposure in rare minnows (Wei et al., 2008).

In this study, PFDoA induced oxidative stress and ultimately led to oxidative damage. Increased lipid peroxidation following PFDoA exposure may be attributed to the induction of ROS, which enhance oxidation of polyunsaturated fatty acids and lead to lipid peroxidation (Liu et al., 2007). In these zebrafish, the basal levels of MDA were restored in the lowest dose group (20 μg/g); however, as the concentration of PFDoA increased and the time elapsed since injection increased, a significant irreversible induction of lipid peroxidation occurred.

As a result of this oxidative stress, fish, like many other vertebrates, try to reduce the damage using the antioxidant defense system. GSH is an important antioxidant, the content of which could reflect the antioxidant potential of the organelle, while the antioxidant enzyme GPx catalyzes the reduction of hydrogen peroxide at the expense of GSH. In our study, PFDoA induced a marked dose-dependent decrease in GSH content 48 h post-injection, and this depletion was accompanied by a significant reduction in SOD and CAT activity as well as a dramatic dose-dependent increase in GPx activity. The antioxidant enzyme CAT catalyzes the decomposition of endogenously produced hydrogen peroxide. SOD, the endogenous scavenger, catalyzes the dismutation of the highly reactive superoxide anion to H_2O_2 (Husain and Somani, 1998). Therefore, alteration of these parameters indicated the presence of a high level of ROS, suggesting that oxidative stress-related processes were involved in the PFDoA-induced *in vivo* toxicity.

The observed significant reduction in SOD and CAT activity in all PFDoA treatment groups at 48 h may result from the high doses of

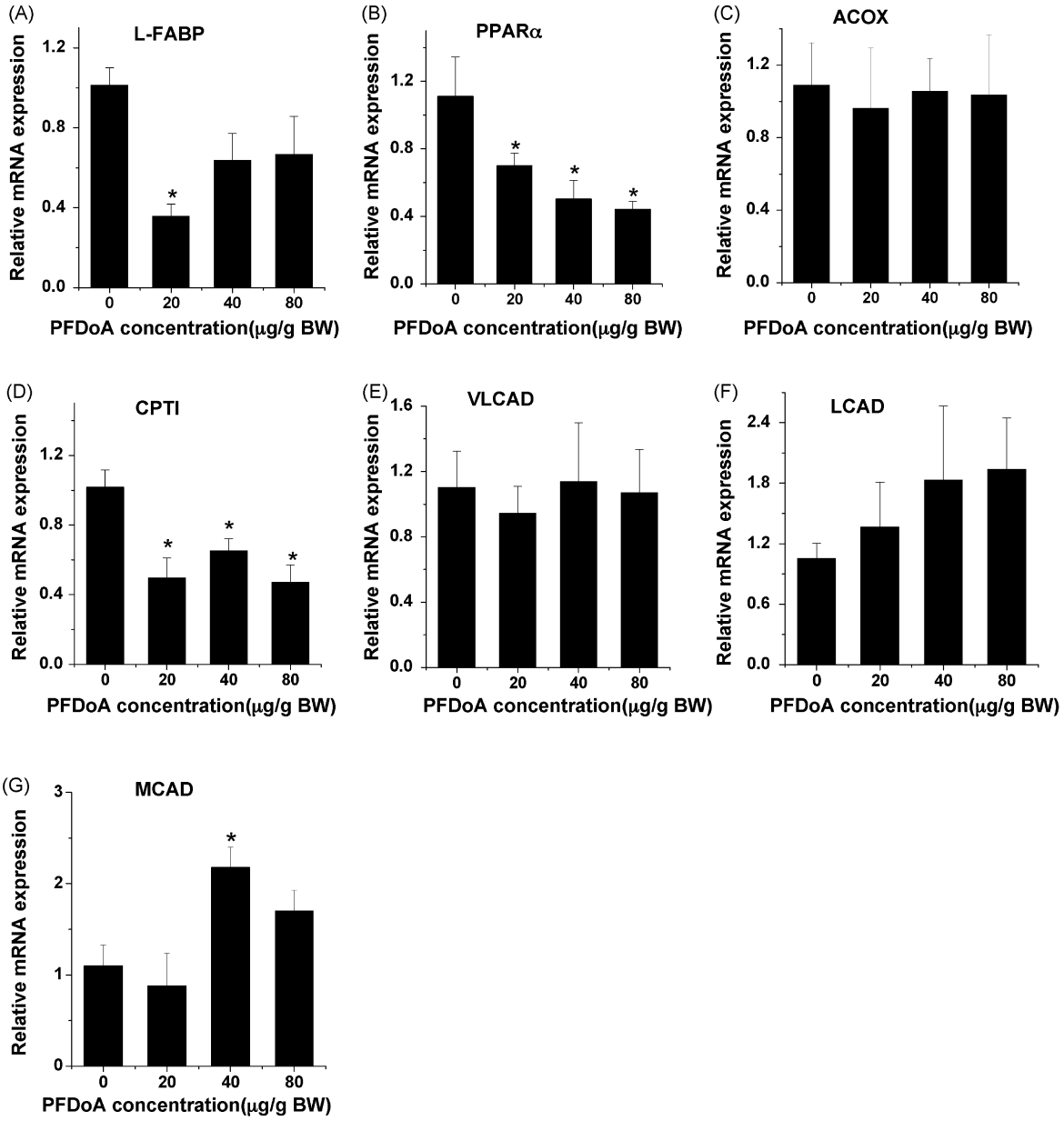

Fig. 4. Real-time PCR analysis of hepatic mRNA expression levels of (A) L-FABP, (B) PPARα, (C) ACOX, (D) CPT I, (E) VLCAD, (F) LCAD, and (G) MCAD in female zebrafish seven days post-acute PFDoA treatment. Gene expression levels represent the relative mRNA expression compared to the control. Values are presented as the mean ± S.E. of six individual fish per group. Significance is indicated by * for $p < 0.05$ compared to the control.

PFDoA as previously demonstrated (Prieto et al., 2007). In addition, the decrease in CAT activity may also be attributed to the excess of superoxide anion radicals that result from the reduction in SOD activity. Indeed, previous studies indicated that high production of superoxide anion radicals inhibits CAT activity (Moreno et al., 2005). CAT and GPx, which both function as scavengers of hydrogen peroxide, presented reverse trends following PFDoA treatment. These distinct reactions reflected the selective effect of the two enzymes in PFDoA-induced oxidative stress. Alternatively, GPx may be more potent in the liver (Kaushik and Kaur, 2003).

Furthermore, time-dependent variation indicated that changes of enzyme activity preceded the increase in lipid peroxidation. Notably, no discernable lipid peroxidation induction was observed at 48 h post-injection, while all the antioxidant parameters changed dramatically at this time point, indicating the contribution of the antioxidant defense system. In fact, the antioxidant enzyme levels were generally affected 48 h after PFDoA injection, and the changes in enzyme activity became insignificant or even opposite (such as SOD activity) at the 96-h and seven-day time points. These time-dependent alterations following a low-dose exposure may result from rapid elimination of PFDoA from organisms (Martin et al., 2003) and an adaptive response of the antioxidant enzyme activity induced by PFDoA treatment.

In order to investigate the ROS production and hepatotoxicity in more detail, the transcriptional effects of PFDoA were also investigated. The disturbance of fatty acid oxidation and the resultant impairment of mitochondrial function may play a role in PFDoA-induced oxidative stress. The transcription of PPARα, which is the main regulator of fatty acid β-oxidation, was inhibited, and this inhibitory effect became more severe with increasing PFDoA concentrations. Several studies have shown significant activation of PPARα by PFOA/PFOS (Maloney and Waxman, 1999; Takacs and

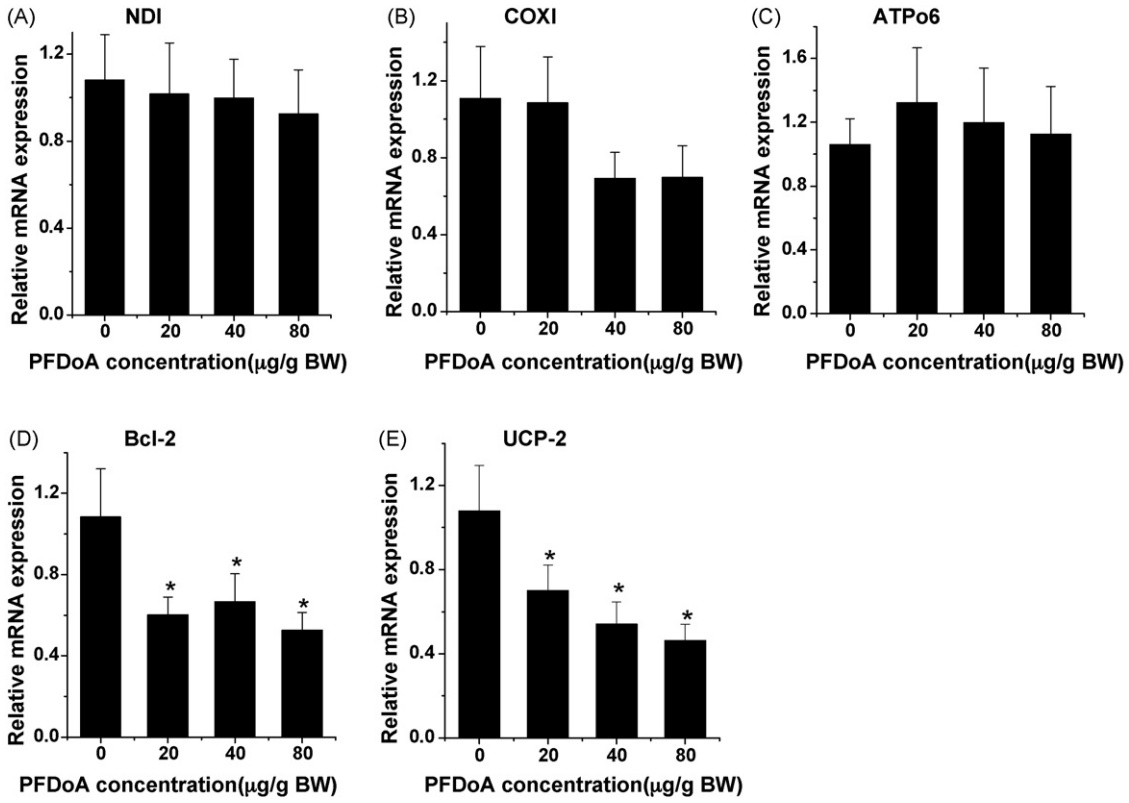

Fig. 5. Real-time PCR analysis of hepatic mRNA expression levels of (A) NDI, (B) COXI, (C) ATPo6, (D) Bcl-2, and (E) UCP-2 in female zebrafish seven days post-acute PFDoA treatment. Gene expression levels represent the relative mRNA expression compared to expression of the control gene. Values are presented as the mean ± S.E. of six individual fish per group. Significance is indicated by * for $p < 0.05$ compared to the control.

Abbott, 2007; Vanden Heuvel et al., 2006). A profiling study in chickens, however, failed to detect PFOS-induced alterations in PPARα (Yeung et al., 2007), and the activation of PPARα by PFDoA in rats was found only at low PFDoA doses (Zhang et al., 2008). Moreover, a recent study conducted on the transcriptional effects of PFOS on isolated hepatocytes of Atlantic salmon revealed that a low dose of PFOS inhibited the transcription of PPARα (Krovel et al., 2008). Together, these results suggest that the effects of PFAAs on PPARα is species-specific or may be dependent on carbon length of PFAAs. Similar to PPARα, the transcriptional levels of CPT I, which is a PPARα target gene that catalyzes the primary rate-limiting step in fatty acid mitochondrial β-oxidation, was also decreased. This result was inconsistent with results obtained from rats (Zhang et al., 2008) but was in agreement with a study conducted on PFOA-treated rare minnows (Wei et al., 2008). Depression of CPT I expression leads to decreased entry of fatty acids and mimics into the mitochondria. Hepatic levels of L-FABP, an intracellular lipid carrier protein, correlate with the rate of peroxisomal β-oxidation (Appelkvist and Dallner, 1980). *In vitro*, PFOA and several other PFAAs interfered with the binding of fatty acids or other endogeneous ligands to rat L-FABP (Luebker et al., 2002). In the present study, PFDoA significantly depressed the mRNA expression of L-FABP only in the low-dose group. The rate-limiting enzyme in fatty acid peroxisomal β-oxidation is ACOX, and the expression and enzyme activity of this enzyme are always used as a biomarker for peroxisome proliferation (Hashimoto, 1999; Reddy and Hashimoto, 2001). Many studies have reported the induction of ACOX expression or enzyme activity in PFAAs-treated rats (Badr and Birnbaum, 2004; Zhang et al., 2008), however, no discernable differences in the mRNA expression of ACOX were observed in PFDoA-treated zebrafish. Thus, in zebrafish, PFDoA exerted an insignificant effect on peroxisomal β-oxidation or peroxisome proliferation. This result was consistent with previous studies conducted in fish, which demonstrated that fathead minnow exhibited a relative unresponsiveness in fatty acid oxidase enzyme activity to PFOA and other mammalian peroxisome proliferators (Scarano et al., 1994; Oakes et al., 2004).

VLCAD, LCAD, and MCAD are chain-length-specific acyl-CoA dehydrogenases that catalyze the chain-specific dehydrogen reaction of fatty acids in mitochondria (Eaton et al., 1996). VLCAD catalyzes the initial step of mitochondrial β-oxidation of long-chain fatty acids, while the subsequent shortened chain length fatty acids are dehydrogenated by LCAD. LCAD-deficient mice exhibit hepatic steatosis, supporting a key role for this enzyme in mitochondrial fatty acid oxidation (Zhang et al., 2007a). Furthermore, MCAD catalyzes a pivotal reaction in mitochondrial fatty acid β-oxidation involving medium-chain fatty acids that enter mitochondria by diffusion, products of mitochondrial β-oxidation of long-chain fatty acids, and products of peroxisomal β-oxidation of long-chain and very long-chain fatty acids (Gulick et al., 1994). In our experiments, PFDoA did not affect the mRNA levels of VLCAD, and the induction of LCAD was not significant. The transcription of MCAD, however, was significantly upregulated. The inconsistent alteration in the expression of MCAD and CPT I observed in the present study suggests complex transcriptional regulation of genes involved in fatty acid β-oxidation following PFDoA exposure. One possible interpretation may be that a dramatic increase of extramitochondrial fatty acid mimics leads to the depression of CPT I to prevent entry of toxic fatty acid mimics into mitochondria. This decrease in CPT I concomitantly inhibits the entry of necessary fatty acids, resulting in decreased energy supply. Thus, the activated expression of MCAD may function as compensation for the energy depletion. Although this possibility is feasible, further research is required to fully elucidate the inconsistencies.

In addition to turbulent fatty acid β-oxidation, ROS generation is also tightly related to the mitochondrial respiratory chain. Cai et al. (1996) reported the induction for mitochondrial respiratory chain components in PFOA-treated rats. Specifically, COXI and NDI were highly responsive to PFOA treatment (4.5- and 24.5-fold increases, respectively). The microarray analysis of PFOA-treated rare minnows also detected altered expression in genes involved in oxidative phosphorylation (Wei et al., 2008). In the present study, PFDoA treatment did not induce apparent changes in the transcriptional expression of COXI, NDI, and ATPo6; yet, the expression of UCP-2 and Bcl-2 was significantly inhibited. UCP-2, which regulates fatty acid metabolism and ROS production (Brand et al., 2004; Klingenberg et al., 2001), facilitates proton leakage across the inner membrane. This leakage modulates mitochondrial function by reducing the membrane potential and uncoupling oxidation phosphorylation to reduce ATP synthesis (Jezek and Garlid, 1998). Previous studies also demonstrated that PFAAs increase proton conductance to uncouple mitochondrial respiration (Starkov and Wallace, 2002). Thus, the observed decrease of UCP-2 may be interpreted as negative feedback to diminish the proton leakage induced by PFDoA. Alternatively, this decrease may be attributed to the downregulation of PPARα, as UCP-2 is a target gene of PPARα (Zhang et al., 2007b). Nonetheless, decreased expression of UCP-2 relieves energy depletion and may enhance the production of ROS, resulting in severe oxidative stress. In addition, in accord with previous reports, UCP-2 may be activated by free fatty acids (Nubel and Ricquier, 2006). Thus, the suppressed UCP-2 expression may reflect a decrease in the content of free fatty acids. This notion is consistent with the decreased entry but enhanced oxidation of fatty acids in mitochondria that was evidenced by the decreased CPT I and enhanced MCAD mRNA levels following PFDoA treatment. Finally, Bcl-2 transcription was significantly inhibited following PFDoA treatment. Bcl-2, which localizes to sites of free radical generation, functions as an apparent antioxidant against oxidative stress to prevent apoptosis (Hockenbery et al., 1993). Interestingly, the alterations in Bcl-2 transcription were consistent with the changes in GSH content. This phenomenon was also observed in previous studies which focused on the gene function of Bcl-2 (Saitoh et al., 2003). Thus, the decreased mRNA levels of Bcl-2 accounted for the enhanced oxidative damage, and this finding further illuminates the depletion of the antioxidant potential.

5. Conclusion

In conclusion, acute PFDoA exposure led to apparent pathological liver damage, elicited oxidative stress, and overwhelmed the homeostasis of antioxidant systems. In addition, PFDoA treatment significantly altered the transcription of genes involved in mitochondrial fatty acid oxidation and antioxidation in zebrafish. Enhanced mRNA expression of MCAD but decreased expression of CPT I, UCP-2, and Bcl-2 contributed to the overloaded generation of ROS, to the collapse of the antioxidant system, and ultimately, to oxidative damage.

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