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Induction of oxidative stress and related transcriptional effects of perfluorononanoic acid using an in vivo assessment

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ABSTRACT

Perfluorononanoic acid (PFNA) is an organic pollutant ubiquitous in the environment. However, the potential toxicity of PFNA remains largely unknown in teleost fish. This study defined the oxidative stress and related transcriptional effects of PFNA at various concentrations on zebrafish larvae. Activities of superoxide dismutase were induced in PFNA-treated groups but attenuated with exposure to higher concentration. Catalase activity and lipid peroxidation were significantly inhibited or increased at the highest concentration, respectively. To test the apoptotic pathway, several genes related to cell apoptosis were examined using real-time PCR. The expression of p53, apoptosis-inducing factor (AIF) and c-Jun NH (2)-terminal kinase (JNK) was partially increased, while Bcl-2, an anti-apoptotic gene, was reduced, with no significant effects on Bax and caspase-3 during the exposure period. The effect of PFNA on lipid β-oxidation system was investigated by examining the activity of peroxisome fatty acyl-COA oxidase (ACOX) and the expression of peroxisome proliferating activating receptors (PPARs). ACOX activity was moderately elevated with marginal significance and was not a significant consequence of PPARα and PPARγ expression. The overall results suggest that turbulence of oxidative stress and apoptotic pathway is involved in PFNA-induced toxicity in zebrafish larvae, and the gene expression patterns are able to reveal some potential mechanisms of developmental toxicity.

1. Introduction

Perfluoroalkyl acids (PFAAs) are a family of perfluorinated chemicals that consist of a carbon backbone with hydrogen replaced by fluorine. The high energy C-F bonds result in remarkable chemical stability and are highly resistant to degradation. Their wide application in industry and common consumer products over the past several decades has resulted in persistent and widespread pollution in the environment, including air, water and soil, as well as in wildlife and humans (Sinclair et al., 2006; Calafat et al., 2007; Lau et al., 2007; Ishibashi et al., 2008; Kunacheva et al., 2012). PFAAs can cause a number of toxic effects in the liver, immune system, and nervous system. They are especially toxic during development and reproduction and have induced cancer (Lau et al., 2007; Johansson et al., 2008). Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) are the two primary PFAA components with C_8 chain lengths. In 2002, PFAAs and their precursors with C_8 chain lengths were phased out of use in consumer products. Consequently, emissions of PFOS and PFOA have decreased (Lau et al., 2007). However, hundreds of related chemicals remain unregulated and perfluorononanoic acid (PFNA), a PFAA with a nine-carbon backbone, continues to be released into the environment worldwide. PFNA has been detected in the environment and in the tissues of humans and wildlife with mean concentrations increasing every year (Calafat et al., 2007; Ishibashi et al., 2008). Accordingly, concerns about the possible health impacts of PFNA exposure have arisen.

Aquatic ecosystems serve as the ultimate sink for many environmental pollutants that accumulate in fish species. The concentrations of PFAAs in fish can reach 8850-fold greater than those in surface water (Sinclair et al., 2006). Oxidative damage may play an important role in the developmental toxicity caused by PFAAs in fish. PFAA-induced oxidative stress has been reported in rare minnow (Wei et al., 2008; Liu et al., 2009), zebrafish (Liu et al., 2008; Shi et al., 2008; Shi and Zhou, 2010), Japanese medaka (Yang, 2010), salmon (Arukwe and Mortensen, 2011), tilapia (Liu et al., 2007) and so on. While several toxicity tests have shown that PFAAs can cause oxidative stress as measured by various end points, changes to oxidative parameters and related genes are variable by species and treatment regimen. PFOA induces a significant inhibition of catalase (CAT) activity at high concentrations with no changes of superoxide dismutase (SOD) or glutathione peroxidase (GPx) activities in the liver of male Japanese medaka (Yang, 2010). Liu et al. (2007) reported significant induction of caspase-3, -8, and -9 activities accompanied by increased levels of SOD, CAT activities and lipid peroxidation (LPO) level (measured by maleic dialdehyde, MDA) in PFOA treated hepatocytes of freshwater tilapia, whereas GPx activity was decreased. In contrast, Atlantic salmon cells exposed to 25.0 mg/L PFOS for 48 h significantly down-regulate caspase 3B expression. Low concentration of PFOS (2.1 mg/L) inhibits the

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transcription of peroxisome proliferating activating receptor α (PPARα) although PFOS acts as a PPARα agonist at high concentration (25.0 mg/L) (Krøvel et al., 2008). Peroxisome fatty acyl-COA oxidase (EC 1.3.3.6; ACOX) activity in the liver of fathead minnows increases with low PFOA concentrations but is attenuated with exposure to higher PFOA concentrations (Oakes et al., 2004). PFOA increases PPARα mRNA levels in the gill of rare minnows while no obvious change in PPARα expression is observed in the liver (Liu et al., 2009). Another study confirms that exposure of salmon to PFOA produce changes in mRNA expression of PPARs and ACOX1, but these responses show marked organ differences (Arukwe and Mortensen, 2011). It's essential to gain more knowledge about the action modes of PFAAs to fully elucidate the mechanisms of PFAA toxicity.

Compared to shorter chain PFAAs, PFNA has a higher accumulation propensity and is more physiologically persistent (Kudo et al., 2001; Ohmori et al., 2003). The biological effects and toxicities of PFNA are similar to other PFAAs and include hepatotoxicity, developmental and reproductive toxicity, immunotoxicity, hormonal effects and carcinogenicity in rodent (Lau et al., 2007; Fang et al., 2010). PFNA seemed to be more toxic than PFOA based on 72 h LC_{50} using a zebrafish assay (Zheng et al., 2012). To date, only limited studies about the toxic mechanisms of PFNA have been performed in aquatic organisms (Zhang et al., 2012; Zheng et al., 2012). Until today no study describes the PFNA-induced oxidative effects in teleost fish. With the potential developmental disrupting potency of PFNA, more attention should be paid to the toxicity of PFNA.

Given the experimental advantages, including small size of the embryo, cheap maintenance, readily available, well-documented biological and genetic information, and many mutants, the zebrafish embryo is considered to be an ideal model for studying toxicological mechanisms. Zebrafish embryo assay is also regarded to be a pain-free in vivo test and is gradually being accepted as a good replacement for other types of animal experiments (Langheinrich et al., 2002; Yang et al., 2009). In this study, zebrafish embryos were used to analyze the mechanism of PFNA-induced oxidative toxicity. The changes in the activities of antioxidative enzymes and lipid peroxidation products were measured. Gene expression patterns related to the apoptosis pathway (e.g., pro-apoptotic [p53, Bax, JNK, AIF, caspase-3] and anti-apoptotic [Bcl-2]) were also examined to elucidate the potential mechanism of apoptosis induced by PFNA. The effect of PFNA on the lipid β-oxidation system was investigated by examining the expression of PPARs, which has been shown to be peroxisome proliferator inducible, and the activity of the ACOX enzyme, whose transcription is induced by PPARs.

2. Materials and methods

2.1. Chemicals

PFNA (97% purity) was obtained from Sigma-Aldrich Chemical Corporation (St. Louis, MO, USA). The stock solution (15,000 mg/L) was prepared by dissolving the crystal in chromatography-grade dimethyl sulfoxide (DMSO). This was stored at 4 °C. Exposure solutions were diluted from the stock solution with embryonic water (0.2 mM $Ca(NO_3)_2$, 0.13 mM $MgSO_4$, 19.3 mM NaCl, 0.23 mM KCl and 1.67 mM HEPES, pH = 7.2) to create the final concentrations. Concentrations of DMSO in the exposure solutions were less than 0.01% (v/v).

2.2. Zebrafish maintenance and embryo exposure

Adult zebrafish were maintained at 28 ± 0.5 °C in a closed flow-through system with charcoal-filtered tap water. Aquaria were cleaned each week. The fish were fed live *Artemia* nauplii twice daily. Zebrafish embryos were obtained from spawning adults in groups of about 6 males and 3 females in tanks overnight. Mating, spawning, and fertilization took place within 30 min after light onset in the morning as has been previously used. Nylon nets were used at the bottom of each tank to allow eggs to settle and prevent adult fish from consuming them.

The zebrafish develop rapidly and gastrulation begins at about 6 hpf (hours post fertilization). Spontaneous movements emerge and tail is detached from the yolk with heart beating at 24 hpf, and embryogenesis is essentially completed at 96 hpf, as most organs are formed and functioning by this time (Kimmel et al., 1995). Thus at 6 hpf embryos were examined under a dissecting microscope, and those embryos that developed normally and reached the blastula stage were selected for PFNA exposure. Embryos were placed in an incubation chamber at 28 ± 0.5 °C and subjected to a 14/10 h light/dark cycle.

2.3. Activities of antioxidant enzymes

After PFNA treatment at various concentrations (0, 0.2, 0.5, 1, 5, and 15 mg/L) for 140 h, the exposure solution was removed, and the larvae were rinsed gently with distilled water three times. Whole-body homogenates in 500 μL of cold phosphate buffered saline (PBS, pH = 7.5) were immediately prepared and centrifuged at 12,000 *g* for 15 min at 4 °C. The supernatants were collected for various assays.

Catalase (EC 1.11.1.6; CAT) and superoxide dismutase (EC 1.15.1.1; SOD) activities were measured according to the protocols of commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). CAT activity was measured by the decrease in absorbance at 240 nm due to H_2O_2 consumption. One unit of CAT activity was defined as the amount of enzyme decomposing 1 μmol H_2O_2 in 1 s; SOD activity was evaluated by the inhibition rate of the superoxide radicals-dependent cytochrome C reduction. The result of this enzymatic assay was given in units of SOD activity per milligram of protein (U/mg), where 1 U of SOD was defined as the amount of sample causing 50% inhibition of cytochrome C reduction.

2.4. Lipid peroxidation and protein assay

Lipid peroxidation (measured as MDA) level was determined using a kit (Nanjing Jiancheng Bioengineering Institute). The MDA level was expressed as nanomoles per milligram protein. Protein content was assayed by the Bradford method, using bovine serum albumin (Sigma-Aldrich) as a standard.

2.5. Enzyme activity of ACOX

The assay for ACOX activity (EC 1.3.3.6) was measured according to the method described previously (Oakes et al., 2003). The ACOX activity was expressed as nmol H_2O_2/min/mg protein. Twenty larvae per sample were pooled to measure the ACOX activity.

2.6. Total RNA extraction and quantitative real-time PCR (QPCR)

Total RNA was extracted from 20 zebrafish larvae for each group using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the instructions of manufacturer. RNA integrity was assessed by standard denaturing agarose gel electrophoresis. Real-time PCR was performed with an ABI 7500 fast Sequence Detector System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) using SYBR Premix ExTaq™ (Takara Bio., Japan). The gene-specific primers were used as previously reported (Shi et al., 2008; Deng et al., 2009; Shi and Zhou, 2010) or designed by Primer Premier 5.0 software. The expression level of the target gene was normalized to the mRNA content of its reference gene (β-actin). The melting curve was analyzed to differentiate the desired amplicons and the primer-dimers or DNA contaminants. The gene expression levels were measured in triplicate for each treatment.

2.7. Statistical analysis

All results were presented as mean ± SEM. Differences were evaluated by one-way analysis of variance (ANOVA) and Tukey's post hoc test using SPSS (version 16.0). A p-value less than 0.05 was considered to be significant.

3. Results

3.1. Activities of antioxidative enzymes

SOD, the endogenous scavenger, catalyzes the dismutation of the highly reactive superoxide anion to H_2O_2. In PFNA treated zebrafish larvae, SOD was significantly increased by 67% and 83% in the 1 and 5 mg/L exposure groups, respectively, compared with the control. The SOD was decreased in the 15 mg/L PFNA exposure group (Fig. 1).

CAT is responsible for the reduction of hydrogen peroxide and protection from the oxidation of unsaturated fatty acids in the cell membrane. CAT activity was significantly decreased by 39% in the 15 mg/L exposure group (Fig. 1).

3.2. Lipid peroxidation

MDA was indicative of lipid peroxidation. MDA content was increased by 100.9% in the 15 mg/L exposure group compared with the control. The MDA content was slightly increased but not statistically significant in the 5 mg/L PFNA exposure group ($p > 0.05$). No marked changes were observed when the larvae were exposed to lower concentrations (Fig. 2).

3.3. Effects of PFNA exposure on mRNA expression of genes related to apoptosis

Expression profiles of genes related to apoptosis were measured. c-Jun NH (2)-terminal kinase (JNK) gene transcription was significantly increased by 2.9- and 3.1-fold in the 1 and 5 mg/L PFNA exposure groups, respectively. The increase in 0.5 mg/L treatment group was not significant ($p > 0.05$) (Fig. 3A). The mRNA levels of the p53 gene were significantly elevated in a concentration dependent manner by 1.75-, 2.95- and 3.75-fold in 0.5, 1, and 5 mg/L PFNA exposure groups, respectively (Fig. 3B). Bax and Bcl-2 are two members of the Bcl2 family that play important roles in the regulation of apoptosis. Bax, the multidomain pro-apoptotic gene, exhibited no significant difference between the control and PFNA-treated groups (Fig. 3C). Down-regulation of Bcl-2 (inhibitor of Bax) gene expression was observed upon exposure to 0.5, 1, and 5 mg/L PFNA, decreasing 1.53-, 1.38-, and 1.92-fold, respectively (Fig. 3D). Caspase-3, a critical gene downstream in the apoptosis pathway, exhibited no marked difference relative to the control group (Fig. 3E). Apoptosis-inducing factor (AIF), an initiator of caspase-independent apoptosis, was induced in PFNA treated larvae. Gene transcription was significantly increased by 1.8- and 2.1-fold in the 0.5 and 5 mg/L PFNA exposure groups, respectively (Fig. 3F).

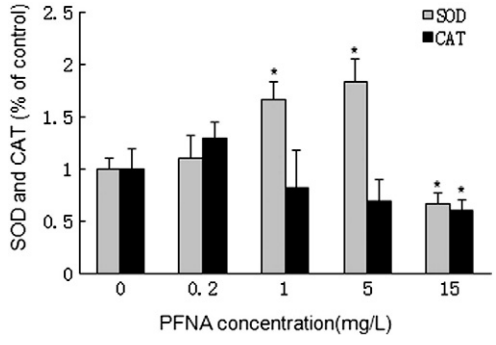

Fig. 1. Alteration of SOD and CAT activities in zebrafish larvae exposed to different concentrations of PFNA. Statistically significant values are indicated by asterisks (*$p < 0.05$). Values are the means of four replicate exposures and are presented as mean ± SEM.

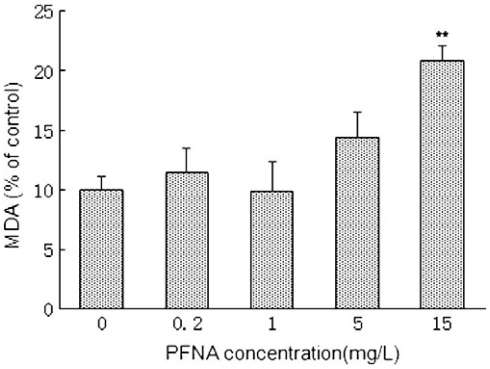

Fig. 2. Changes of MDA level in zebrafish larvae exposed to different concentrations of PFNA. Double asterisk (**) indicates a highly significant difference ($p < 0.01$). Values are the means of four replicate exposures and are presented as mean ± SEM.

3.4. Effects of PFNA exposure on acyl-CoA oxidase activity and PPAR expression

ACOX activity was measured to determine if PFNA exposure was associated with the induction of peroxisome proliferation in zebrafish. The activities of ACOX increased at all concentrations, but the differences were not statistically significant ($p > 0.05$). In the highest concentration (5 mg/L), ACOX activity was increased by 28% with a marginal significance ($p = 0.06$) (Fig. 4A).

PPAR is a key factor in regulating the activity of ACOX in peroxisomes. The expression of PPARα (a and b) and PPARγ was quantified in zebrafish, and the levels of these transcripts are shown in Fig. 4B. PPARαa and b were reduced in 1.0 and 5.0 mg/L PFNA exposure group, respectively, and no different changes were observed in other concentrations. The levels of PPARγ mRNA slightly increased at all concentrations compared to the control group, but the changes were not statistically significant ($p > 0.05$).

4. Discussion

Peroxisomal-oxidation is a process that generates hydrogen peroxide (H_2O_2), which can cause oxidative stress and oxidative damage to proteins and DNA. Gene expression profiles of animals exposed to PFOS and PFOA have revealed that one of the major pathways affected by PFAAs is peroxisomal fatty acid beta-oxidation (Hu et al., 2005; Guruge et al., 2006; Yeung et al., 2007). It is clear from these studies that perturbation of lipid metabolism is one of the main causative biochemical events that lead to the adverse health outcomes associated with PFOS and PFOA exposure. Peroxisome proliferating activating receptor (PPAR) is the principle mediator of peroxisome proliferation and plays a pivotal role in controlling peroxisomal fatty acid oxidation (Mandard et al., 2004). PFAA exposure reportedly produces reactive oxygen species (ROS) and enhances oxidative damage via PPAR activation (Fang et al., 2010; Yang, 2010; Arukwe and Mortensen, 2011). ACOX, which catalyzes the first and rate-limiting step in fatty acid peroxisomal oxidation, is regulated by PPARs. Our data indicated that ACOX activity was moderately influenced by PFNA exposure in zebrafish and was not a significant consequence of PPAR expression. There are conflicting data regarding the ability of PFAAs to induce expression of PPAR in different organisms. For example, while the up-regulated expression of both PPARα and PPARγ is characteristic findings in rats treated with perfluorododecanoic acid (PFDoA) (Zhang et al., 2008) or PFNA (Fang et al., 2010), the inhibition of PPARα by PFDoA (Liu et al., 2008) or PFNA is found in zebrafish (Zhang et al., 2012). PFAAs seem to produce

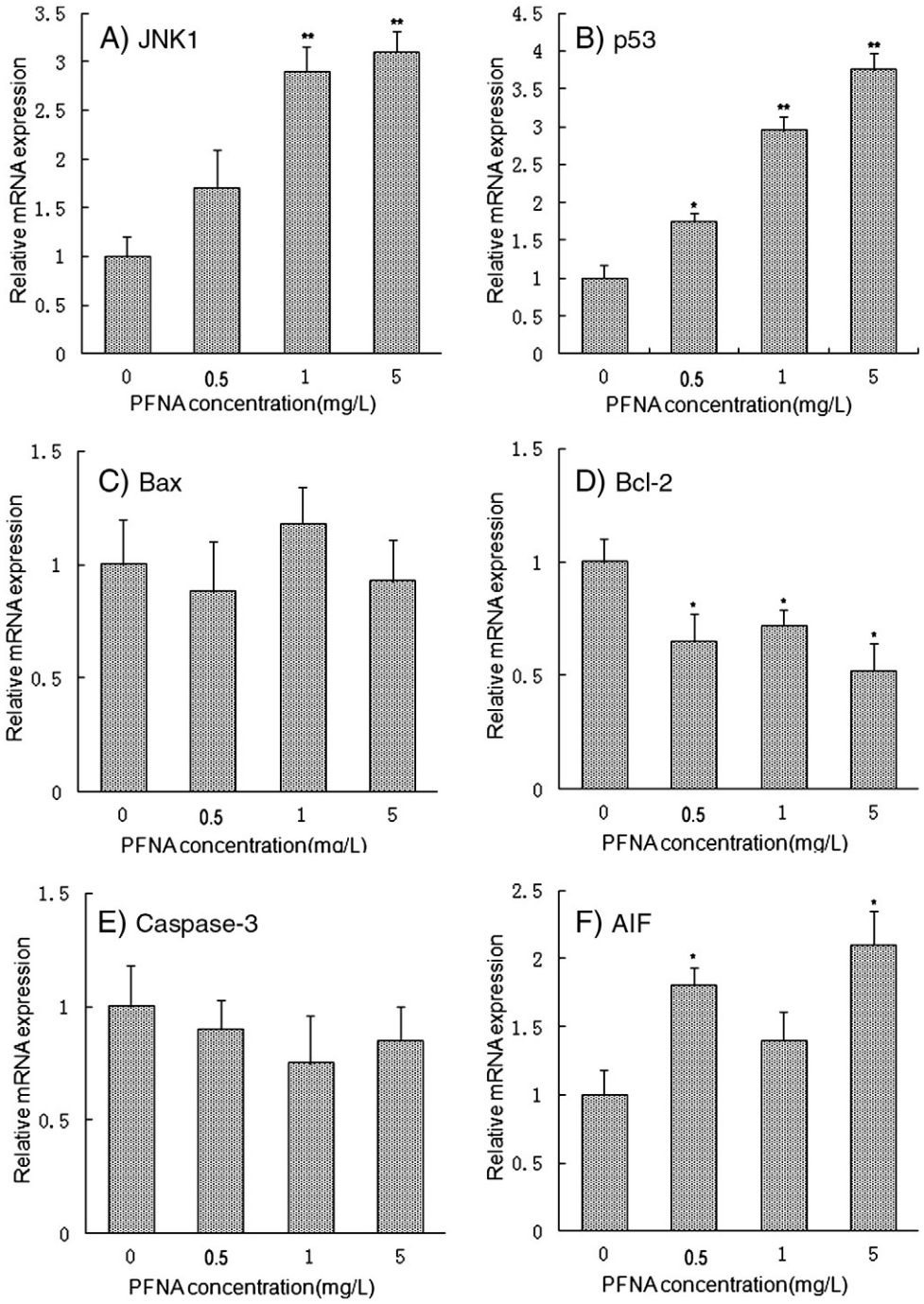

Fig. 3. Quantitative real-time PCR analyses of gene expression levels of (A) JNK1, (B) p53, (C) Bax, (D) Bcl-2, (E) caspase-3, and (F) AIF in zebrafish larvae exposed to different concentrations of PFNA. Gene expression levels represent the relative mRNA expression compared to the control. Values significantly different from the control are indicated by asterisks (*$p < 0.05$; **$p < 0.01$). Values are the means of three replicate exposures and are presented as mean ± SEM.

more robust transcriptional responses than fish. Changes to PPARs and ACOX may also be variable in different tissues and with various treatment concentrations (Oakes et al., 2004; Krøvel et al., 2008; Liu et al., 2009; Arukwe and Mortensen, 2011). In addition, PPAR expression may also change in a stage-specific manner. It has been shown that effects of peroxisome proliferation, including ACOX in goldfish, disappear with the time of exposure through the adaptive response (Mimeault et al., 2006). PFOS elicits a transcriptional response of the PPAR pathway in *Oryzias melastigma* in a stage-specific manner. PPARα and PPARβ are first inhibited at 4 dpf and are induced at 10 dpf (Fang et al., 2012). The molecular mechanism underlying the variable expression pattern is unknown. Although many studies have indicated that the toxicity of PFAAs is related to the activation of PPARs, the present results suggest that the PPAR-independent pathways altered by PFNA, such as the constitutive androstane receptors and the pregnane X receptors involve in xenobiotic metabolism and phase I, II, and III processes, should not be discounted.

Increased hydrogen peroxide (H_2O_2) production due to exaggerated fatty acid β-oxidation may be harmful for the organism (Bonekamp et al., 2009). These may cause deleterious cellular effects, such as cell death and other pathological conditions, as a result of ROS produced during chemical or oxidative stress conditions (Bonekamp et al., 2009). An adaptation during limited oxygen conditions requires the induction of antioxidant and associated enzymes, such as CAT, SOD and

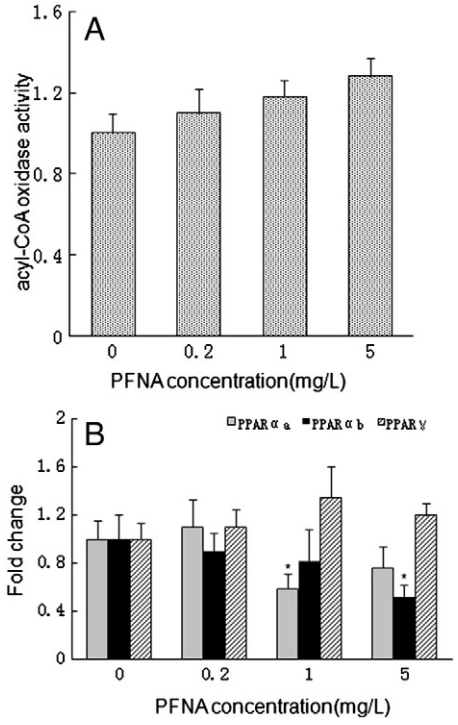

Fig. 4. Peroxisomal acyl-CoA oxidase activity (A) and gene expression levels of PPARs (B) in zebrafish larvae exposed to different concentrations of PFNA. Values significantly different from the control are indicated by asterisks ($^*p < 0.05$). Gene expression levels represent the relative mRNA expression compared to the control and are the means of three replicate exposures presented as mean ± SEM.

GPx, in order to reduce potential damage during oxygen reintroduction. In the present study, SOD was significantly induced in PFNA-treated groups. Increased SOD activity may be responsible for alleviation of PFNA-caused oxidative stress in zebrafish. However, higher concentration of PFNA appeared to reduce SOD activity. This may be explained by the excess production of superoxide radicals, which, after their transformation to H_2O_2, cause an oxidation of cysteine in the enzyme and deactivate SOD activity. This study showed that PFNA induced a significant inhibition of CAT activity at high concentration. This result is in accordance with the decreased catalase enzyme activity in the liver of Japanese medaka (Yang, 2010) and the oxidative stress detected in male fathead minnow (Oakes et al., 2004) after exposure to PFOA. The decrease in CAT activity may be attributed to the excess of superoxide anion radicals that result from the reduction in SOD activity. In fact, previous study indicated that high production of superoxide anion radicals inhibited CAT activity (Moreno et al., 2005). Catalase serves as a primary defense enzyme against oxidative stress such as hydrogen peroxide generated from peroxisomal β-oxidation of fatty acids. If catalase is a limiting step in the removal of peroxide, this can result in an increase in hydrogen peroxide, which can induce lipid peroxidation, membrane damage, accumulation of lipofuscin, and DNA damage. Indeed, a significant irreversible induction of lipid peroxidation occurred at high exposure concentration in our study. This showed that although antioxidant status played an important protective role against PFNA-caused oxidative stress, the defense capacity cannot sufficiently alleviate oxidative damage occurring under high exposure concentration.

Stress-induced apoptosis is thought to contribute to abnormal development during embryogenesis. In the present investigation, several important genes that might be involved in apoptosis were investigated to elucidate the mechanisms of PFNA exposure. JNK serves as an important pro-apoptotic mechanism in oxidative stressed cells, and mitochondria are the main sites of action for JNK during apoptosis (Shen and Liu, 2006). The tumor suppressor gene, p53, has been examined for its role in DNA damage-induced apoptosis that is chemically induced (Shen and White, 2001; Langheinrich et al., 2002). The alteration of the pro-apoptotic genes suggests that p53 and JNK are involved in cell apoptosis due to PFNA exposure. Bcl-2, which is localizes to sites of free radical generation, functions as an apparent antioxidant against oxidative stress to prevent apoptosis. The Bax/Bcl-2 ratio appears to be a critical determinant of the integrity of mitochondrial membrane (Shen and White, 2001). The increased Bax/Bcl-2 ratio suggests that disruption of mitochondrial membrane integrity may occur after PFNA exposure. Caspase-3 has been identified as a key executor of apoptosis and is one of the most important caspases to be activated downstream in apoptosis pathways (Cohen, 1997). AIF is an initiator of caspase-independent apoptosis. The gene expression pattern indicates that waterborne PFNA is able to induce apoptosis through the involvement of caspase-independent pathway in zebrafish larvae. This is consistent with a study in rodents, which reveals that PFNA induces apoptosis in a caspase-independent death pathway (Fang et al., 2010). On the basis of results from the present study, it may be hypothesized that PFNA exposure induces oxidative stress and causes damage to cell membranes (as evident from increased LPO levels) and subsequently, the oxidative stimulus may trigger JNK and p53. JNK represses the function of the anti-apoptotic gene Bcl-2. The resulting increase of Bax/Bcl-2 ratio disrupts the mitochondrial membrane. Once the integrity of the mitochondrial membrane is disrupted, mitochondrion releases cytochrome C and triggers AIF, therefore inducing caspase-independent apoptosis.

Thus, oxidative stress and the activation of apoptotic signaling pathway contribute to PFNA-induced toxicity in zebrafish. The gene expression patterns in the larvae reveal potential toxicity mechanisms of PFNA. As the post-hatched larval stage is more sensitive than the adult stage, sensitivity to the chemical can be increased with chronic exposure. It is therefore necessary for future studies to investigate the extended post-hatched stage in addition to the life-cycle exposure to fully investigate the potential toxicity of PFNA.

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