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Lipid accumulation responses in the liver of *Rana nigromaculata* induced by perfluorooctanoic acid (PFOA)

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ABSTRACT

Perfluorooctanoic acid (PFOA) is a perfluorinated compound that is widely distributed, is persistent in the environment, and has a low-level chronic exposure effect on human health. The aim of this study was to investigate the peroxisome proliferator activated receptors γ (PPARγ) and the sterol regulatory element-binding protein 2 (SREBP2) signaling pathways in regulating the lipid damage response to PFOA in the livers of amphibians. Male and female frogs (*Rana nigromaculata*) were exposed to 0, 0.01, 0.1, 0.5 and 1 mg/L PFOA. After treatment, we evaluated the pathological changes in the liver by Oil Red O, staining and examined the total cholesterol (T-CHO) and triglyceride (TG) contents. The mRNA expression levels of *PPARγ*, *Fatty acid synthase (FAS)*, *Acetyl-CoA carboxylase (ACC)*, *Glycerol-3-phosphate acyltransferase (GPAT)*, *SREBP2* and *3-hydroxy-3-methylglutaryl CoA (HMG-CoA)* were measured by quantitative real-time polymerase chain reaction (qRT-PCR). The administration of PFOA caused marked lipid accumulation damage in the amphibian livers. The T-CHO contents were elevated significantly after PFOA treatment; these results show a dose-dependent manner in both sexes. The TG content showed a significant increase in male livers, while it was elevated significantly in female livers. The RT-PCR results showed that the mRNA expression levels of *PPARγ*, *ACC*, *FAS*, *GPAT*, *SREBP2* and *HMG-CoA* were significantly dose-dependently increased in the PFOA-treated groups compared with those of the control group. Our results demonstrated that PFOA-induced lipid accumulation also affected the expression levels of genes *FAS*, *ACC*, *GPAT* and *HMG-CoA* in the PPARγ and SREBP2 signaling pathways in the liver. These finding will provide a scientific theoretical basis for the protection of *Rana nigromaculata* against PFOA effects.

1. Introduction

Amphibians, as indicators of environmental and ecological safety, are threatened. The attenuation of amphibian populations is an environmental problem that has been attracting interest since the first report by Wake in *Science* on the worldwide amphibian decline (Palen and Schindler, 2010; Wake, 1991). Moreover, the rate of amphibian declines will continue to rise and accelerate in the future (Stuart et al., 2004). A growing body of evidence has shown that environmental pollutants are in some way responsible for amphibian declines (Hayes et al., 2010). Persistent organic pollutants (POPs) are a matter of concern. POPs are capable of migrating in various environmental media and accumulate in wildlife; these pollutants are highly toxic, even at low ecologically relevant concentrations (El-Shahawi et al., 2010). Many studies have reported the toxic effects of POPs on amphibians (Ankley et al., 2004; Mikkelsen and Jenssen, 2006). Among POPs, polyfluoroalkyl chemicals (PFCs) received great attention (Renner, 2001).

PFCs are used commonly in commercial applications, such as surfactants and insecticides (Lee et al., 2013). PFCs are persistent in the environment and are resistant to degradation. PFCs are widely distributed in waters and have emerged as global environmental pollutants

Abbreviations: PFOA, perfluorooctanoic acid; PPARγ, peroxisome proliferator activated receptors γ; SREBP2, sterol regulatory element-binding protein 2; T-CHO, total cholesterol; TG, triglyceride; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; GPAT, glycerol-3-phosphate acyltransferase; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; qRT-PCR, quantitative real-time polymerase chain reaction; POPs, persistent organic pollutants; PFCs, polyfluoroalkyl chemicals; LPO, lipid peroxidation; H_2O_2, hydrogen peroxide

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(Yamashita et al., 2008). Perfluorooctanoic acid (PFOA) is one of the predominant PFCs found among environmental media and accumulates in biota, such as birds (Kannan et al., 2002), rats (Cui et al., 2009), and fish (Peng et al., 2010), and in human serum (Holzer et al., 2008). PFCs pose a potential environmental risk, especially to aquatic organisms (Peng et al., 2010). Given their highly permeable skins, amphibians are highly sensitive to environmental pollutants (Quaranta et al., 2009). The hepatotoxicity of PFCs has been observed extensively in mammals and fish (Liu et al., 2007; Wang et al., 2015). However, the effects of PFCs in amphibian livers are poorly understood.

Toxicological effect studies on the hepatotoxicity of PFCs have extended from mammalian systems to lower vertebrates, such as fish (Du et al., 2009). In rats, toxicological experiments and toxicokinetics suggest that PFCs mainly accumulate in the liver (Cui et al., 2009; Kudo et al., 2007). Similar effects have yet to be revealed in Chinese sturgeons, in which concentrations of perfluorinated acids in the liver are 10–100 times greater than in other organs (Peng et al., 2010). Through accumulation in organisms, PFCs pose a potential environmental risk. Rats were administered with PFOA or PFOS solutions by gavage for 28 days, and histopathological observation showed serious liver damage, including hepatocytic hypertrophy, cytoplasmic vacuolation, fatty degeneration and focal hemorrhage (Cui et al., 2009). PFOA induced reactive oxygen species generation and increased lipid peroxidation (LPO) levels in the primary hepatocytes of freshwater tilapia (*Oreochromis niloticus*) (Liu et al., 2007). Numerous studies show that PFCs can activate hepatic peroxisome proliferator-activated receptors (PPARs), which are lipid metabolism regulators; this metabolism can be described by the physiological processes of lipogenesis, lipid oxidation, and lipid secretion (Luebker et al., 2002; Yang and Li, 2007; H. Zhang et al., 2013; L. Zhang et al., 2013). Perfluorododecanoic acid (PFDoA) exposure diminished the absolute weight of livers, increased the LPO levels, significantly decreased serum triglyceride (TG) concentrations, and induced the hepatotoxicity of lipid homeostasis disruption in rat livers (Zhang et al., 2008). For Atlantic salmon hepatocytes, perfluorooctane sulfonamide evokes deleterious effects on cellular lipid homeostasis and, consequently induced significant changes in the fatty acid (FA) composition (Wagbo et al., 2012). Exposure to PFOS affected the growth, survival and hepatotoxicity in zebrafish and rats (Du et al., 2009; Cui et al., 2009). However, a pathologic assessment of PFOA exposure in amphibian livers has yet to be conducted, and the underlying mechanism of PFOA-induced liver injury remain unknown.

Here, our study focused on PFOA-induced liver damage, and we hypothesized that PFOA-induced liver injury may be associated with PPARγ and SREBP2 signaling pathway-associated genes. In our previous study, *Rana nigromaculata* were used to assess microcystin-LR toxic effects on the reproductive system (Jia et al., 2014; H. Zhang et al., 2013; L. Zhang et al., 2013). However, we have little knowledge about the effects of PFOA exposure on the expression of PPARγ and SREBP2 in the liver tissues of amphibians. Therefore, this study selected *Rana nigromaculata* as a model to identify the underlying mechanisms related to liver damage associated with PFOA exposure.

2. Materials and methods

2.1. Chemicals

PFOA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Total cholesterol (T-CHO) and TG enzyme-linked immunosorbent assay (ELISA) kits were purchased from Nanjing Jiancheng Bioengineering Inc. (Nanjing, Jiangsu, China). The TRIzol® Plus RNA Purification Kit (CAS: 12183-555) and the SuperScript™ III First-Strand Synthesis SuperMix for quantitative real-time polymerase chain reaction (RT-PCR) (CAS: 11752-050) were purchased from Invitrogen. The RNase-Free DNase Set (CAS: 79254) was purchased from Qiagen. The Power SYBR® Green PCR Master Mix (CAS: 4367659) was purchased from Applied Biosystems.

2.2. Animals and treatment conditions

Healthy adult frogs (*Rana nigromaculata*) were captured from the suburbs of Zhejiang ChangXing Creative Ecological Agriculture Development Co., LTD (Huzhou, Zhejiang, China). The frogs were fed *Eisenia fetida* twice a day. Two-hundred healthy frogs (100 male, 100 female) adapted to the condition for 1 week and were randomly divided into five groups (n = 20 per group). They were exposed to 0, 0.01, 0.1, 0.5 and 1 mg/L of PFOA solution to a depth of 3 cm for 14 days. The doses were based on a previously report about PFOA exposure and were equivalent to 0.2-, 5-, 10- and 20-fold the concentration found in *R. nigromaculata* (Loos et al., 2008). The day after the last treatment, all frogs were treated with pithing, and then the livers were quickly harvested. At the end of the experiment, the samples were immediately fixed for subsequent experiments. The study protocol was approved by the local government and all animals were handled in accordance with the guidelines set by the Experimentation Ethics Review Committee. Animal treatment conditions were described in detail in the Supplemental material (S1).

2.3. Oil Red O staining

Liver tissues were sectioned, frozen, and then fixed in neutral formalin for 15 min. The frozen sections were then stained with Oil Red O and restained with hematoxylin for 1 min. Finally, the sections were mounted with glycerogelatin and photographed under a microscope.

2.4. T-CHO content measurement

Cholesterol, which was produced from cholesterol esterase cleavage, was converted to cholesteric enone and hydrogen peroxide (H_2O_2) by cholesterol oxidase. H_2O_2 reacted with 3,5-DHBS and 4-aminoantipyrine and produced red quinone compounds, which were used to measure the optical density in a spectrophotometer at 510 nm. The color depth of the produced quinone compounds is directly proportional to the content of T-CHO. The T-CHO content was measured in accordance with the instructions provided by the commercial detection kits.

2.5. TG content measurement

In the presence of lipoprotein lipase, TGs were hydrolyzed to glycerol and FAs. Glycerol with glycerol kinase was phosphorylated to glycerol-3-phosphate, which was involved in a redox reaction where H_2O_2 was produced. Finally, red quinone compounds were generated in a reaction of H_2O_2, 4-chlorophenol and 4-aminoantipyrine and were analyzed by peroxidase. The color depth of the produced quinone compounds is directly proportional to the TG content, which was determined by using commercial detection kits in a spectrophotometer at 510 nm.

2.6. Quantitative RT-PCR

A TRIzol® Plus RNA Purification Kit was used for total RNA extraction from the frog livers. Total RNA was isolated according to the manufacturer's instructions and was detected using an ultraviolet spectrophotometer. The reverse transcription experiment was completed by PCR gene amplification (Bio-Rad, USA). A quantitative PCR primer was designed using the Primer Premier 6.0 and Beacon designer 7.8 software, followed by gene synthesis (Table 1). The expression levels of the target genes were calculated using the $2^{-\triangle\triangle Ct}$ method, where Ct represents the threshold cycle. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Each group was analyzed in triplicate. The RT-PCR program was reported by our previous studies (Jia et al., 2014; Tang et al., 2016), and more detailed steps are available in the Supplementary materials (S2).