



# Perfluorononanoic acid-induced apoptosis in rat spleen involves oxidative stress and the activation of caspase-independent death pathway

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## ARTICLE INFO

### Article history:

Received 14 September 2009

Received in revised form 14 October 2009

Accepted 19 October 2009

Available online 28 October 2009

### Keywords:

PFNA

Spleen

Apoptosis

Oxidative stress

AIF

## ABSTRACT

Perfluoroalkyl acid (PFAA)-induced apoptosis has been reported in many cell types. However, minimal information on its mode of action is available. This study explored the possible involvement of apoptotic signaling pathways in a nine-carbon-chain length PFAA—perfluorononanoic acid (PFNA)-induced splenocyte apoptosis. After a 14-day exposure to PFNA, rat spleens showed dose-dependent levels of apoptosis. The production of pro-inflammatory and anti-inflammatory cytokines was significantly increased and decreased, respectively. However, protein levels of tumor necrosis factor receptor 1 (TNFR1), fas-associated protein with death domain (FADD), caspase 8 and caspase 3, which are involved in inflammation-related and caspase-dependent apoptosis, were discordant. Peroxisome proliferator-activated receptors alpha (PPAR $\alpha$ ) and PPAR $\gamma$  genes expression was up-regulated in rats treated with 3 or 5 mg/kg/day of PFNA, and the level of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) increased concurrently in rats treated with the highest dose. Moreover, superoxide dismutase (SOD) activity and Bcl-2 protein levels were dramatically decreased in spleens after treatment with 3 and 5 mg/kg/day of PFNA. However, protein levels of Bax were unchanged. Apoptosis-inducing factor (AIF), an initiator of caspase-independent apoptosis, was significantly increased in all PFNA-dosed rats. Thus, oxidative stress and the activation of a caspase-independent apoptotic signaling pathway contributed to PFNA-induced apoptosis in rat splenocytes.

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## 1. Introduction

Perfluoroalkyl acids (PFAAs) represent a class of chemicals composed of a hydrophobic perfluorinated alkyl chain and a hydrophilic anionic functional group. These unique properties contribute to their widespread use in a variety of commercial products, such as household surface finishes, food packaging, water- and stain-resistant materials and fire-fighting foams (Kissa, 2001). The high-energy C–F bond resists both biological and chemical degradation, making PFAAs highly persistent in the environment (Giesy and Kannan, 2002). The occurrence of organic fluorine in human serum was first reported in the 1960s (Taves, 1968), and human biomonitoring of the general population for PFAAs in breast milk, liver, seminal plasma, and umbilical cord blood serum were carried out in succession after 2000 (Lau et al., 2007; Apelberg et al., 2007; Guruge et al., 2005). In recent years, studies have shown that the serum concentrations of perfluorononanoic acid (PFNA), which contains nine carbon chains, was the third most frequently detected PFAA, after perfluorooctanesulfonate (PFOS) and perfluoro-

rooctanoate (PFOA) (Hart et al., 2009; Calafat et al., 2007; Yeung et al., 2009; Kärman et al., 2009). One reason for the high concentration of PFNA detected in organisms is that some fluoropolymer industries use PFNA instead of PFOS and PFOA as a processing aid in the manufacture of fluoropolymers, such as polyvinylidene fluoride, since perfluorooctanesulfonyl-based chemicals were phased out by the 3M Company (St. Paul, MN, USA) between 2000 and 2002 (Prevedouros et al., 2006). Another reason for this is that the longer-chain PFAAs showed more bioaccumulation than other shorter-chain homologs (Alexander and Olsen, 2007).

The biological effects and toxicities of PFNA are similar to other PFAAs and include hepatotoxicity, developmental toxicity, reproductive and immunotoxicity, hormonal effects and carcinogenicity in rodent (Kudo et al., 2000; Vanden Heuvel et al., 2006; Lau et al., 2007). Both PFOA and PFOS can promote apoptosis in human normal or carcinoma cell lines, monkey kidney-derived Vero cells and fish primary cultured hepatocytes (Hu and Hu, 2009; Fernández Freire et al., 2008; Kleszczyński et al., 2007; Liu et al., 2007). However, little information on PFAA-induced apoptosis and related apoptotic signaling pathways is available in rodents.

PFOA exposure has been reported to change the gene expression of pro-inflammatory cytokines in the mouse spleen (Son et al., 2008), and PFNA elevated the level of interleukin-1 (IL-1) in

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rat serum (Fang et al., 2009). Pro-inflammatory cytokines are well-known regulators of apoptosis (Muppidi et al., 2004; Cnop et al., 2005). For example, the binding of pro-inflammatory cytokines to their receptors can trigger caspase-dependent apoptosis. However, PFOA and PFOS have been reported to promote hepatoma Hep G2 cells apoptosis even though the mRNA level of caspase 3 remained unchanged (Hu and Hu, 2009). In addition, Atlantic salmon cells exposed to 25.0 mg/l PFOS for 48 h significantly down-regulated caspase 3B expression (Krøvel et al., 2008). Thus, the roles of pro-inflammatory cytokines and caspase proteins in PFAA-induced apoptosis need to be further investigated.

The other apoptotic signaling pathway, which is independent of caspase recruitment, is initiated by mitochondrial outer membrane (MOM) permeabilization and involves the release of intermembrane mitochondrial proteins, such as endonuclease G (Endo G) and apoptosis-inducing factor (AIF), into the cytosol. These proteins are then translocated to the nucleus (Susin et al., 2000). Once in the nucleus, AIF acts in a cooperative manner with other factors to promote nuclear apoptosis. A few pro-apoptotic stimuli, e.g., oxidative stress, can regulate the permeabilization of MOM. In addition, Bcl-2 family proteins play important roles in the permeabilization of MOM during the early stage of apoptosis (Jourdain and Martinou, 2009). PFOA exposure reportedly produced reactive oxygen species (ROS) and enhanced hepatic oxidative damage via peroxisome proliferator-activated receptor (PPAR) activation in rodent (Badr and Birnbaum, 2004; Cai et al., 1995). PFAA exposure also changes the expression of Bcl-2 in hepatoma Hep G2 cells (Hu and Hu, 2009). Thus, oxidative stress, Bcl-2 family proteins and AIF may be involved in PFAA-induced apoptosis.

To test the above hypothesis and identify a specific mode of action for PFNA-induced apoptosis, male rats were continuously exposed to PFNA for 14 days. Splenocyte apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL). The levels of pro-inflammatory and anti-inflammatory cytokines, and hydrogen peroxide ( $H_2O_2$ ), as well as the activity of superoxide dismutase (SOD) were measured in spleen tissue homogenates. The expression of PPAR genes and proteins related to apoptotic signaling pathways were investigated.

## 2. Materials and methods

### 2.1. Animals

Male Sprague–Dawley (SD) rats (220–230 g) were obtained from the Weitong Lihua Experimental Animal Center (Beijing, China). Animals were housed singly and maintained in a mass air displacement room with a 12:12 h light:dark cycle at 20–26 °C and a relative humidity of 40–60%. Food and water were provided ad libitum throughout the study. After 1 week of adaptation, the rats were separated into four groups of six rats each. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

### 2.2. Reagents and treatments

PFNA (acid, CAS number 375-95-1, 97% purity) was purchased from Sigma–Aldrich (St. Louis, MO). All other chemicals and reagents were of analytical grade. Anti-caspase 8 rabbit mAb, anti-caspase 3 rabbit mAb, anti-Bcl-2 rabbit mAb, anti-Bax rabbit mAb and anti-phospho-c-Jun NH<sub>2</sub>-terminal protein kinases (JNK) (Thr183/Tyr185) rabbit mAb were purchased from Cell Signaling Technology (Beverly, MA). Anti-tumor necrosis factor receptor 1 (TNFR1) rabbit mAb, anti-fas-associated death domain (FADD) rabbit mAb, anti-CD3 rabbit mAb and anti-AIF rabbit mAb were purchased from Abcam (Cambridge, UK).

PFNA was suspended in 0.5% Tween-20 in water (Beijing Chemical Reagent Co., Beijing, China) daily and given via gavage to rats at doses of 1, 3, or 5 mg/kg body weight/day for 2 weeks. Control rats were treated similarly but given vehicle only. The doses were based on a previous study in which half of the mice died when treated with 10 mg of PFNA/kg/day for 14 days (Fang et al., 2008). After 14 days of treatment, rats were euthanized by decapitation. Spleens were immediately isolated and weighed. One part of the spleen was fixed in 10% neutral-buffered formalin for TUNEL examination; the other was immediately frozen in liquid nitrogen and stored at –80 °C until further use.

### 2.3. TUNEL

The TUNEL assay was performed using In Situ Cell Death Detection Kit/POD (Roche, Mannheim, Germany) to identify double-stranded DNA fragmentation. Briefly, tissue slides were deparaffinized, rehydrated, and then quenched in 3% hydrogen peroxide for 10 min at room temperature before treating with 20 µg/ml proteinase K for 15 min at 37 °C. After rinsing twice with phosphate-buffered saline (PBS), the slides were incubated at 37 °C for 60 min with the diluted TUNEL reaction mixture. The slides were then treated with 3% bovine serum albumin (BSA) blocking solution for 25 min at room temperature and incubated with the secondary antifluorescein-POD-conjugate for 30 min. After washing four times in PBST (0.01 M PBS, 0.1% Tween-20), diaminobenzidine chromogenic reagent was applied to the sections. The sections were then counterstained with hematoxylin, dehydrated in a graded series of ethanol, and cleared in xylene. Apoptotic cells were detected manually at a 40× magnification under a Nikon Eclipse 50i light microscope.

### 2.4. Cytokine analysis

Concentrations of TNF-α, IL-1, IL-6, IL-10, and γ-interferon (IFNγ) were measured in homogenates from frozen spleen tissue by enzyme-linked immunosorbent assays (ELISAs), in accordance with the manufacturer's directions (Biosource International, Camarillo, CA). Absorbances were measured with an ELISA plate reader (Multiskan Ex Primary EIA V. 2.3).

### 2.5. Determination of $H_2O_2$ concentration and SOD activity

Spleen homogenates (10% and 1%) were used to assay for  $H_2O_2$  concentration and SOD activity, respectively, using kits according to the user's manual (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). 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. Protein concentration was assayed using the Bradford protein assay kit (Tiangen, Beijing, China), using BSA as a standard.

### 2.6. Quantitative real-time PCR

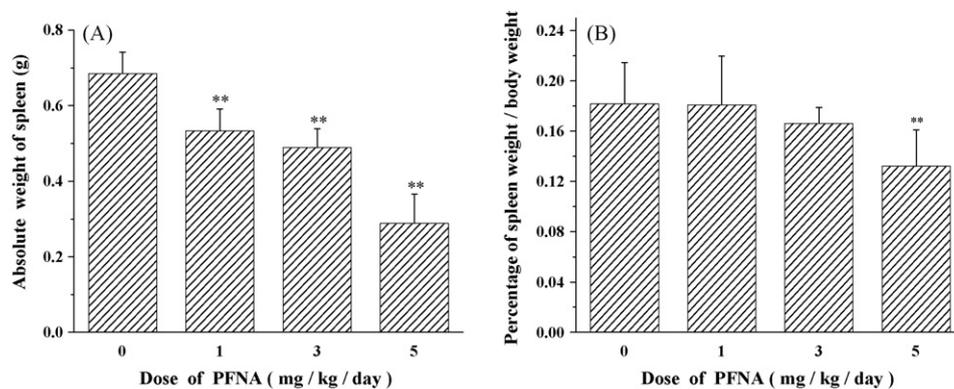
RNA was extracted from the spleen homogenates using the RNeasy minikit (Qiagen, Hilden, Germany). First-strand cDNA was prepared with a reverse transcription system (Promega, Madison, WI). Quantitative real-time PCR was performed on a Stratagene Mx3000p qPCR system (Stratagene, Cedar Creek, USA). SYBR Green PCR Master Mix reagent kits were used according to the manufacturer's instructions for quantification of gene expression. 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, 9 µl nuclease-free water, and 1 µl of cDNA template. Rat-specific primers were designed as follows for specific genes: PPARα forward primer: TGAAGATTCGGAACTGC; PPARα reverse primer: TCCTGCGAG-TATGACCC. PPARγ forward primer: GTTGATTTCTCCAGCATTTTC; and PPARγ reverse primer: TTGATCGCACTTTGGTATT. The β-actin housekeeping gene was used as an internal control. Cycling conditions were as follows: 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Transcripts were quantified by the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001).

### 2.7. Immunoblotting analysis

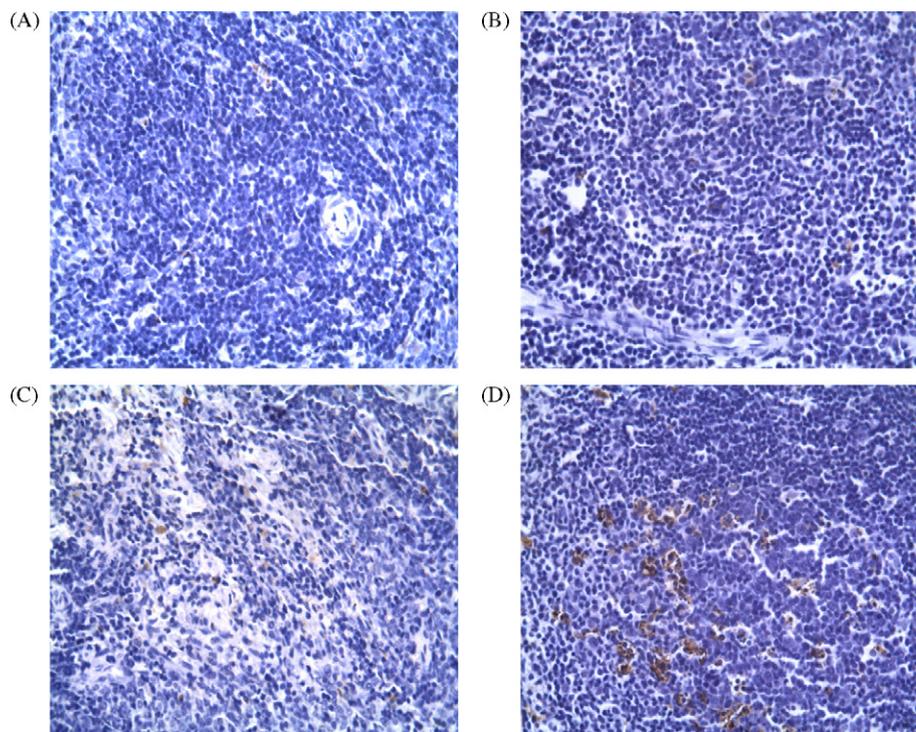
Frozen spleen tissues were excised and homogenized using a tissue homogenizer for 30–60 s on ice in lysis buffer containing a cocktail of protease and phosphatase inhibitors (Appligen Technologies, Inc., China). Lysates were clarified by centrifugation at 12,000 × g for 5 min, and their protein content was determined by the Bradford protein assay (Tiangen, Beijing, China). Approximately 50 µg of total protein was resolved on 10% sodium dodecylsulfate (SDS)-polyacrylamide-gels and then transferred to polyvinylidene fluoride membranes (Amersham Biosciences, Piscataway, NJ). The membranes were blocked for 1 h in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 10% nonfat powdered milk and then incubated overnight at 4 °C in 5% nonfat powdered milk/TBST containing specific primary antibodies. After washing, the membranes were then incubated for 2 h at room temperature with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G, as the secondary antibody. The immunoreactive bands were detected with an ECL Western Blot Detection Kit (Tiangen, Beijing, China), according to the manufacturer's protocol. The intensity of the bands was quantified by densitometry. The samples were normalized to β-actin.

### 2.8. Statistical analysis

Data were expressed as the means ± SE and analyzed using one-way analysis of variance (ANOVA). A Duncan multiple range test was used to identify means that differed significantly ( $p \leq 0.05$ ) between treatment and control groups. All analyses were carried out using SPSS for Windows 13.0 Software (SPSS, Inc., Chicago, IL, USA).



**Fig. 1.** Absolute weight (A) and relative weight (B) of spleens from control and PFNA-exposed male rats. For all panels, the values are the mean  $\pm$  SE for six rats per group. \*\* $p < 0.01$ .



**Fig. 2.** PFNA-induced apoptosis in rat spleen. Representative photomicrographs of TUNEL-positive (brown) apoptotic cells in spleens exposed to control conditions (A), 1 mg of PFNA/kg/day (B), 3 mg of PFNA/kg/day (C), and 5 mg of PFNA/kg/day (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

### 3. Results

#### 3.1. Spleen weight

Rats receiving 1, 3 or 5 mg of PFNA/kg/day showed dose-dependent decreases in the absolute spleen weight (decreased by 22.2%, 28.7% and 57.9%, respectively;  $p < 0.01$ ) compared to the control group. However, the ratio of spleen weight to body weight only significantly decreased (91.5% of the control,  $p < 0.01$ ) in the group given the highest dose (Fig. 1).

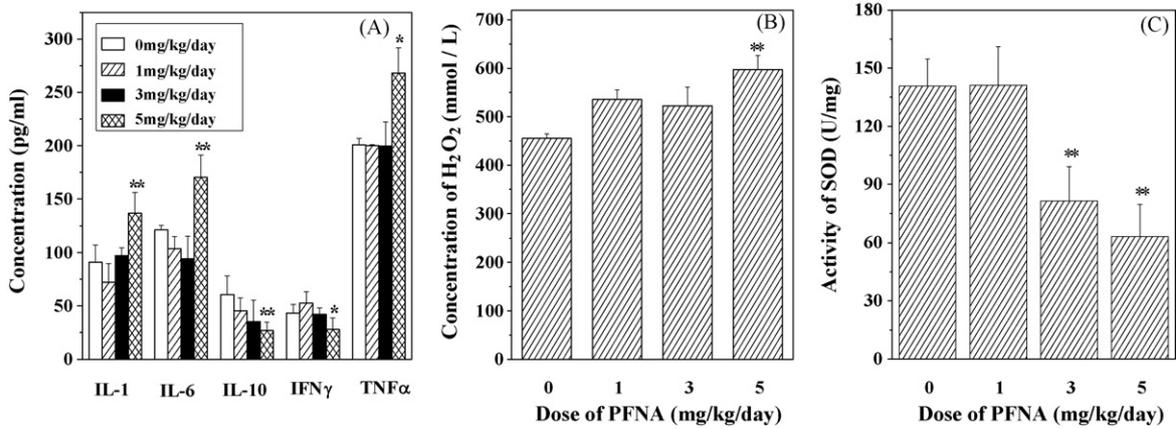
#### 3.2. Lymphoid cell apoptosis

To evaluate the impact of PFNA on lymphoid cell apoptosis, spleen sections were examined for DNA fragmentation indicative of cell death using the TUNEL reaction. Control animals had very few TUNEL-positive cells, indicating very low-level lymphoid cell apoptosis in normal spleens (Fig. 2A). The number of TUNEL-

positive cells was similar to control levels in the 1 mg PFNA/kg/day group (Fig. 2B), but the number of apoptotic cells increased obviously in animals receiving 3 and 5 mg PFNA/kg/day (Fig. 2C and D).

#### 3.3. Cytokine and $H_2O_2$ concentrations and SOD activity

Rats receiving 5 mg/kg/day of PFNA had significantly increased levels of pro-inflammatory IL-1, IL-6, and TNF- $\alpha$  (149.4%, 140.5%, 130.1% of the control, respectively), but pro-inflammatory IFN $\gamma$  and anti-inflammatory cytokine IL-10 levels were decreased by 34.9% ( $p < 0.05$ ) and 56.7% ( $p < 0.01$ ), respectively, compared to control rats (Fig. 3A). Meanwhile, the concentration of  $H_2O_2$  was significantly increased by 31.2% ( $p < 0.05$ ) in the group treated with the highest dose compared to the control group (Fig. 3B). However, SOD activity was significantly decreased by 42.2% and 55.1% in the 3 and 5 mg PFNA/kg/day groups, respectively ( $p < 0.01$ ), compared to the control rats (Fig. 3C).



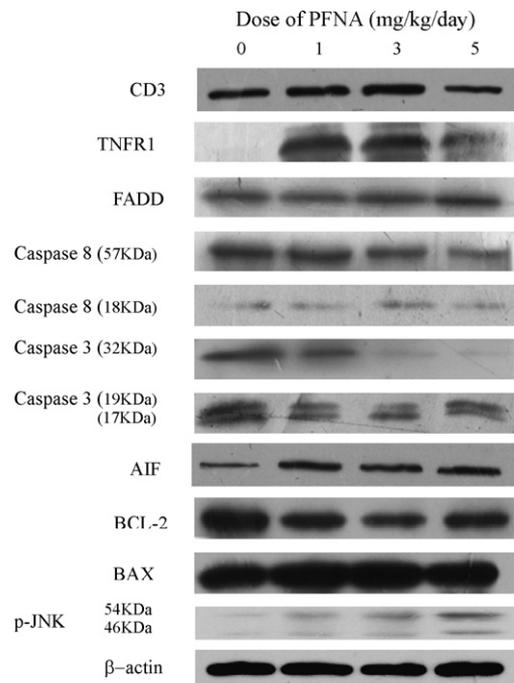
**Fig. 3.** Concentrations of cytokines, H<sub>2</sub>O<sub>2</sub> and the activity of SOD in rat spleen. Concentrations of cytokines (A), H<sub>2</sub>O<sub>2</sub> (B) and the activity of SOD (C) in the homogenates of spleen from control and PFNA-exposed male rats. Values are presented as the mean ± SE for six rats per group. \**p* < 0.05; \*\**p* < 0.01.

**3.4. Gene expression**

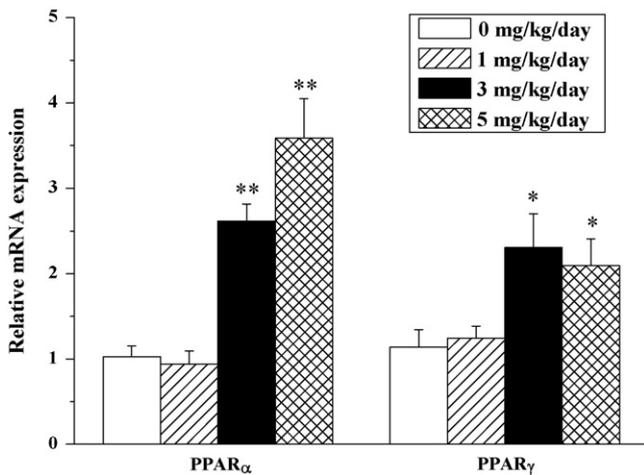
PPAR $\alpha$  expression in rat spleens was 2.6-fold (*p* < 0.01) and 3.6-fold (*p* < 0.01) greater than the control following 3 and 5 mg/kg/day PFNA administration, respectively. In addition, the PPAR $\gamma$  mRNA levels were 2.3-fold (*p* < 0.05) and 2.1-fold (*p* < 0.05) higher than the control in these groups (Fig. 4).

**3.5. Western blotting**

TNFR1 protein expression increased 1.5-fold (*p* < 0.01) and 1.3-fold (*p* < 0.05) in rats that were given 1 and 3 mg/kg/day of PFNA, respectively. AIF expression increased 1.4-fold (*p* < 0.05), 1.56-fold (*p* < 0.01), and 1.6-fold (*p* < 0.01) in rats given 1, 3 and 5 mg/kg/day of PFNA, respectively. The p-JNK level only increased in the group given the highest dose (1.2-fold, *p* < 0.05). Protein expression of precursors of caspase 8 and caspase 3 were decreased (1.3- and 1.4-fold, respectively, *p* < 0.05) in rats receiving 5 mg/kg/day of PFNA, and the expression of cleaved caspase 3 (17 kDa) also decreased (1.5-fold, *p* < 0.01) in the spleens of these rats. Bcl-2 levels decreased by 1.4- and 1.5-fold (*p* < 0.01) in the rats given 3 and 5 mg/kg/day of PFNA, respectively. CD3 expression decreased by 1.3-fold (*p* < 0.05) in response to the highest dose only. The protein levels of cleaved caspase 8 (18 kDa), FADD and Bax were unchanged (Fig. 5).



**Fig. 5.** Analysis of protein expression. Representative western blots are shown in the top panels, and results from densitometry analysis of the blots are shown in the bottom panels. These values are the means ± SE of three independent experiments with similar results.  $\beta$ -Actin was used as the loading control. \**p* < 0.05; \*\**p* < 0.01.



**Fig. 4.** Quantitative real-time PCR analysis of PPAR $\alpha$  and PPAR $\gamma$  expression in spleen mRNA obtained from control and PFNA-exposed male rats. Gene expression levels represent the relative mRNA expression compared to the control gene expression levels. Values indicate the mean ± SE for six rats per group. \**p* < 0.05; \*\**p* < 0.01.

#### 4. Discussion

The immune system can be a target for xenobiotic-induced toxicity (De Jong and Van Loveren, 2007), and it is sensitive to low-level doses of chemicals, even in the absence of toxicity to other organ systems (Sharma and Reddy, 1987). The spleen is the largest lymphoid organ and plays an important role in both innate and adaptive immune responses. Lymphocytes in the spleen are activated when presented with foreign antigens; these activated lymphocytes are sensitive to signals that lead to apoptosis (Gao et al., 2008). This study showed that PFNA exposure caused obvious lymphoid cell apoptosis in the rat spleen, similar to PFNA-induced apoptosis in the mouse spleen (Fang et al., 2008). Cell apoptosis can be promoted by xenobiotics-induced inflammation in organism (Horn et al., 2000), and PFOA exposure has been demonstrated to increase the gene expression of pro-inflammatory cytokines in the mouse spleen (Son et al., 2008). Pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-1, and IL-6, play important roles in cellular apoptosis (Muppidi et al., 2004; Cnop et al., 2005). For example, TNF- $\alpha$  binds to TNFR1 and triggers the recruitment of receptor interacting protein (RIP) and TNFR1-Associated Death Domain (TRADD). TRADD recruits FADD, which interacts with caspase 8, leading to activation of pro-caspase 8 and subsequent activation of executioner caspase 3. IL-1 induces apoptosis through the activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) or the mitogen/stress-activated protein kinase (MAPK/SAPK) cascade, i.e., extracellular signal-regulated kinases (ERK 1/2), JNK, and p38 kinases (Eizirik and Mandrup-Poulsen, 2001; Mahr et al., 2000). Moreover, IL-1 and IL-6 enhance TNF- $\alpha$ -induced apoptosis (Mandrup-Poulsen et al., 1987; Saldeen, 2000; Wagley et al., 2007). In this study, all of the detected pro-inflammatory cytokines in spleen were elevated following 5 mg/kg/day PFNA treatment, except for IFN $\gamma$ , which is secreted by T lymphoid cells in the spleen. The significantly decreased expression of CD3, a T cell-specific surface antigen, in the group given the highest dose suggested that the decrease in T cells contributed to the drop in IFN $\gamma$  levels in the spleen. The protein level of p-JNK was correspondingly increased in the spleen of rats receiving the highest dose of PFNA, which was consistent with the occurrence of obvious apoptosis in these rats. In addition, TNFR1 levels increased following PFNA administration, but FADD was unchanged. Unexpectedly, expression of caspase 8 and caspase 3 precursors was decreased, with their cleavage products unchanged and decreased, respectively. A previous study showed significant down-regulation of caspase 3B in primary cultured hepatocytes from Atlantic salmon exposed to 25.0 mg/l PFOS for 48 h, despite a modest level of apoptotic stress (Krøvel et al., 2008). In addition, PFOA and PFOS had no effect on the mRNA level of caspase 3 in hepatoma Hep G2 cells, although the cells showed significant apoptosis (Hu and Hu, 2009). However, perfluorodecanoic acid (PFDA)-induced activation of caspase 9 and caspase 3 in HCT116 cells has been reported (Kleszczyński et al., 2009). Thus, although alteration of pro-inflammatory cytokines and apoptosis were evident following PFNA treatment, the role of caspase proteins in this process was unclear and warrants further exploration.

PFAAs exert most of their biological effects by activating PPARs (Permadi et al., 1992; Klaunig et al., 2003). A recent study demonstrated that splenic responses to PFOS are largely eliminated in PPAR $\alpha$ -null male 129/Sv mice (Qazi et al., 2009), indicating a noticeable role for PPAR in the toxic effect of PFAAs in the rodent spleen. Our data showed that PFNA administration up-regulated expression of both PPAR $\alpha$  and PPAR $\gamma$ . Activation of PPARs increases peroxisomal  $\beta$ -oxidation and produces excess H<sub>2</sub>O<sub>2</sub> in hepatic cells (Badr and Birnbaum, 2004). In this study, PFNA exposure also elevated H<sub>2</sub>O<sub>2</sub> levels in the rat spleen. In addition, SOD, an enzyme that is considered as the first line of defense against oxygen toxicity, was significantly decreased. This suggested that oxygen toxicity occurred in the spleen. Similar to pro-inflammatory cytokines,

H<sub>2</sub>O<sub>2</sub> can induce JNK activation (Torres, 2003), which may contribute to the increased level of p-JNK in this study. JNK serves as an important pro-apoptotic mechanism in oxidatively stressed cells, and mitochondria are the main site of action for JNK in apoptosis (Shen and Liu, 2006). Mitochondria play a central role in apoptosis induced by various stimuli. Perfluorochemical exposure can uncouple mitochondrial respiration (Starkov and Wallace, 2002), disrupt mitochondrial bioenergetics, and lead to caspase-dependent apoptosis (Kleszczyński et al., 2009). However, little information on the mode of action involved is available. Oxidative stress, such as H<sub>2</sub>O<sub>2</sub>, may disrupt mitochondrial membrane potential and release intermembrane proteins (Chandra et al., 2000). In addition, members of the Bcl-2 family of proteins have been associated with the mitochondrial membrane and regulation of its integrity (Gupta, 2003), and p-JNK represses the function of the anti-apoptotic proteins Bcl-2 and Bcl-xl (Yamamoto et al., 1999; Kharbanda et al., 2000). In this study, the expression of Bcl-2 was decreased, and the expression of Bax was unchanged; thus, the Bax/Bcl-2 protein ratio, which appears to be a critical determinant of the integrity of mitochondrial membrane (Shen and White, 2001), was increased following PFNA treatment. The increased H<sub>2</sub>O<sub>2</sub> level and Bax/Bcl-2 protein ratio and the up-regulation of p-JNK suggested that disruption of mitochondrial membrane integrity occurred in splenocytes after PFNA administration. Once the integrity of mitochondrial membrane is disrupted, mitochondria release molecules including cytochrome c and AIF into the cytosol (Van Gurp et al., 2003). Cytochrome c triggers the well-defined, caspase-dependent apoptotic pathway, while AIF induces caspase-independent apoptosis (Wu et al., 2002; Parrish and Xue, 2003). In this study, the level of AIF protein was significantly increased in all rats that received PFNA, which may be responsible for the observed apoptosis.

Previous studies have demonstrated that continuously generated H<sub>2</sub>O<sub>2</sub> inhibited classical apoptosis and initiated an alternate, AIF-dependent process instead (Son et al., 2009; Barbouti et al., 2007). Thus, increased H<sub>2</sub>O<sub>2</sub> level, up-regulated AIF protein, and down-regulated caspase proteins indicated that PFNA-induced apoptosis may largely be due to the oxidative stress and the activation of caspase-independent apoptosis.

In conclusion, subacute exposure of PFNA promoted obvious apoptosis in rat spleens. The increased pro-inflammatory cytokines may contribute to this apoptosis via p-JNK, which subsequently affected the function of Bcl-2 family proteins. However, the role of caspase proteins in this process was inconspicuous. In contrast, increased H<sub>2</sub>O<sub>2</sub> level, decreased SOD activity, and elevated AIF level suggested that oxidative stress and a mitochondria-related, caspase-independent death signal pathway significantly contribute to PFNA-induced apoptosis in rat splenocytes.

#### Conflict of interest

None declared.

#### Acknowledgments

This research was supported by the National Natural Science Foundation of China (Grants# 20837004 and 20777074).

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