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Alterations of Cytokines and MAPK Signaling Pathways are Related to the Immunotoxic Effect of Perfluorononanoic Acid

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Perfluorononanoate (PFNA), a perfluorinated alkyl acid containing nine carbon chains, has been detected in abiotic and biotic matrices worldwide. Although a few studies have reported toxic effects of PFNA, little information of the mechanism has been offered. In this study, the effects of PFNA exposure on thymus and the related mechanisms were investigated. Male rats were orally dosed with 0, 1, 3, or 5 mg PFNA/kg/day for 14 days. A significant decrease of body weight and thymus weight were observed in the rats receiving 3 or 5 mg PFNA/kg/day. Histopathological examination revealed dose-dependent increases in thymocyte apoptosis. Rats receiving 3 or 5 mg PFNA/kg/day exhibited increased interleukin (IL)-1 and decreased IL-2 concentrations in sera, whereas elevated IL-4 and cortisol levels only occurred in the highest dose group. Quantitative real-time PCR indicated that expression of peroxisome proliferator–activated receptor alpha (PPAR-α) was increased in the thymi of all dosed rats, and a similar trend occurred for PPAR-γ in the two highest dose groups. The mRNA levels of c-Jun NH_2-terminal kinase (JNK), nuclear factor-kappa B, p65 subunit, and inhibitory protein IκBα were unchanged; however, increased and decreased mRNA levels of p38 kinase were found in rats exposed to 3 or 5 mg PFNA/kg/day, respectively. Decreased Bcl-2 mRNA levels were observed in rats receiving 5 mg PFNA/kg/day. A significant increase in protein levels of phospho-JNK was found in all PFNA-treated rats. Phospho-p38 was significantly enhanced in 1 and 3 mg PFNA/kg/day groups, whereas phospho-IκBα remained consistent in all rats studied. Together, these data suggested that apart from the activation of PPARs, PFNA exposure in rats lead to the alteration of serum cytokines, which subsequently activated mitogen-activated protein kinase signaling pathways and potentially modulated the immune system. Additionally, increased serum cortisol and decreased expression of Bcl-2 in thymus likely contributed to the PFNA-induced thymocyte apoptosis.

Key Words: **PFNA; immunotoxicity; PPAR; MAPK; NF-κB.**

The perfluorinated alkyl acids (PFAAs), a family of perfluorinated chemicals that consist of a carbon backbone and a charged functional moiety, have been manufactured for over 50 years. Concern about the safety of these chemicals has been propagated by their widespread use, especially in industry and consumer products, and their persistence in the environment (Dinglasan-Panlilio and Mabury, 2006). Due to a phase out of perfluorooctanesulfonyl-based chemistries by the 3M Company (St Paul, MN) between 2000 and 2002, global manufacturing emissions of perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) were precipitously dropped (Hart *et al*., 2008; Lau *et al*. 2007). However, hundreds of related chemicals, homologues with shorter or longer alkyl chain, are not regulated (Jensen and Leffers, 2008). Perfluorononanoate (PFNA), a nine carbon backbone of PFAAs, and ammonium perfluorononanoate (APFN) are primarily used as a processing aid in the manufacture of fluoropolymers such as polyvinylidene fluoride. Based on the production of APFN from 1975 to 2004, estimated historical global manufacturing emissions of APFN are 70–200 tons and no information was found describing efforts to reduce emissions from APFN manufacture (Prevedouros *et al*., 2006). In addition, some precursors such as fluorotelomer alcohols may be subsequently transformed into PFNA during environmental degradation processes, which make this chemical more available to wildlife and humans. For instance, the concentrations of PFNA in northern sea otters increased 10-fold from 2004 to 2007 (Hart *et al*., 2008). PFNA geometric mean concentrations in serum of the U.S. population in 2003–2004 were approximately twice that of levels in 1999–2000 (Calafat *et al*., 2007). Notably, PFNA has also been detected in human seminal plasma (Guruge *et al*., 2005) and milk (Kärrman *et al*., 2007; So *et al*., 2006).

Previous studies suggested that the biological effects and toxicities of PFNA are similar to other PFAAs, for example, the immunotoxicity of both PFNA and other PFAAs including atrophy of lymphoid organs, suppression of lymphoid cell proliferation, decrease of lymphocytes, etc. (Fang *et al*., 2008; Yang *et al*., 2000). Furthermore, PFNA is likely to be more toxic than PFAAs with short carbon chain length, such as PFOA and PFOS (Kleszczyński *et al*., 2007; Ohmori *et al*., 2003). Therefore, to study toxicities of lower dose of PFNA exposure will be more appropriate for evaluating the potentially health risk of perfluorinated chemicals. However, the information of PFNA immunotoxicity is limited. Peroxisome proliferator–activated

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receptors (PPARs) play an important role in the toxic effects of fluorinated chemicals, including immunotoxicity, but the involvement of these receptors is not sufficient to explain all the toxic effects observed in immune system, as seen in PPAR-α-knock out mice exposed to PFOA (Yang *et al*., 2002b). Other potential modes, such as cytokine production or lipid regulation, have been suggested and are the subject of further investigation (DeWitt *et al*., 2009; Yang *et al*., 2002a). Assessment of cytokine expression and production is a promising tool to evaluate the effects of chemical exposure on the immune system (Cohen *et al*., 1999; House, 1999; Vandebriel *et al*., 1998). PFOA potentially elevates the production of interleukin (IL)-4 and augments the IgE in mice response to environmental allergens (Fairley *et al*., 2007). To date, however, little information about the effect of PFAAs on the variation of cytokines and the subsequent immunomodulation triggered by them has been described.

Cytokines exert immunomodulatory effects via regulation of intercellular or intracellular signaling pathways, such as the mitogen-activated protein kinase (MAPK) signaling pathways and nuclear factor-kappa B (NF-κB) signaling pathways (Schmeck *et al*., 2007). Both of these pathways play critical roles in lymphocyte differentiation, maturation, and apoptosis (Sung *et al*., 2006; Trompezinski *et al*., 2008; Wilkinson and Kaye, 2001). PPAR agonists have been reported to activate different MAPK subfamilies depending on the cell type (Gardner *et al*., 2003; Lennon *et al*., 2002), and the activation of MAPK and the down-regulation of the anti-apoptotic gene Bcl-2 expression strongly influence xenobiotic-induced lymphoid cell apoptosis (Kroemer, 1997). On the other hand, PPAR agonist-induced apoptosis can occur through the suppression of NF-κB signaling pathways and the down-regulation of Bcl-2 (Chen *et al*., 2002). Thus, the administration of PFNA, a PPAR agonist (Fang *et al*., 2008), to an animal will likely influence these signaling pathways and subsequently impact the immune system.

In this study, rats were dosed via gavage with 0, 1, 3, or 5 mg PFNA/kg body weight/day for 14 days. The toxicities of PFNA on the thymus were evaluated by weight and histopathological examination. The serum cytokines and cortisol were detected through enzyme-linked immunosorbent assays (ELISA) and radioimmunoassay (RIA), respectively. The involvements of PPAR, MAPK, and NF-κB signaling pathways in the immunomodulation of PFNA were investigated at both gene and protein levels.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats were purchased from Weitong Lihua Experimentary Animal Central (Beijing, China). The rats were housed in polystyrene cages (one rat per cage) and maintained in a controlled environment at 20–26°C with a relative humidity of 30–70% and a 12-h light-dark cycle. Drinking water and food were provided *ad libitum* throughout the study. After a one-week acclimation period, forty rats, with body weights of 320–340 g, were divided into four groups of ten animals each for this study. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

Reagents and treatments. Anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}) rabbit monoclonal antibody (mAb), anti-phospho-SAPK/JNK (Thr^{183}/Tyr^{185}) rabbit mAb, and anti-phospho-IκBα ($Ser^{32}/^{36}$) mouse mAb were purchased from Cell Signaling (Beverly, MA). PFNA (CAS number 375-95-1, 97% purity) was purchased from Sigma Aldrich (St. Louis, MI). All other chemicals and reagents were analytical grade. PFNA was prepared in 0.5% Tween-20 (Beijing Chemical Reagent Co., Beijing, China) and administered orally via gavage to rats in the treatment groups for two weeks at doses of 1, 3, or 5 mg/kg body weight/day. Control rats were treated similarly with vehicle only. The doses were based on previous study in which half of the mice died during the treatment of 10 mg PFNA/kg/day for 14 days (Fang *et al*., 2008). After fourteen days of treatment, the rats were weighed, and six rats from each group were euthanized by decapitation in this study. The others were used for proteomics analysis in the future. Blood collection was conducted in a strict way to avoid handling-induced or circadian rhythm-related variable in cortisol levels according to Pruett's (1999) method. Blood was collected and centrifuged at 2000 × g at 4°C for 15 min. Serum was stored at −80°C until analysis. Thymi were immediately isolated, weighed, and divided into two portions. One part was fixed in 10% neutral buffered formalin for histological examination, and the other part was immediately frozen in liquid nitrogen and stored at −80°C until used for RNA and protein isolation.

Histopathological examination. A portion of each thymus sample was fixed in 10% neutral buffered formalin for histological evaluations. Paraffin-embedded tissue sections were cut and stained with hematoxylin and eosin. Histopathological changes were observed under light microscope.

Serum cortisol and cytokine analysis. Concentrations of cortisol in sera samples were detected by RIA using commercial kits from Beijing North Institute of Biological Technology (China). IL-1, IL-2, and IL-4 were measured by ELISA in accordance with the manufacturer's directions (Biosource International, Camarillo, CA). Absorbance was measured with an ELISA plate reader at wavelengths of 492, 450, and 450 nm, respectively.

Reverse transcription and quantitative real-time PCR analysis. Total RNA was isolated from the thymus using the RNeasy minikit (Qiagen, Hilden, Germany). The concentration was determined using a UV1240 spectrophotometer (Shimadzu, Japan). RNA (1 μg) was used as the template for reverse transcriptase (RT) reactions using an oligo-$(dT)_{15}$ primer (Promega, Madison, WI) and M-MuLV reverse transcriptase (New England Biolabs, UK). The quantitative real-time PCR (qRT-PCR) reactions were performed with the Stratagene Mx3000P qPCR system (Stratagene, La Jolla, CA), and the SYBR Green PCR Master Mix reagent kit (Takala, Dalian, China) was used for quantification of gene expression. Rat-specific primers were designed for the genes of interest: Bcl-2, PPAR-α, PPAR-γ, p65, p38, JNK, and IκBα (Table 1). The housekeeping gene β-actin was used as an internal control, and quantification of the transcripts was performed by the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

Western blotting. Frozen tissue specimens were homogenized using a tissue homogenizer for 30–60 s on ice in lysis buffer containing a protease inhibitor and phosphatase inhibitor cocktail (Applygen Technologies, Inc., China). The protein content was determined using a Bradford Kit (Applygen Technologies, Inc.). Approximately 60 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 2 h in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 10% nonfat dry milk and then incubated overnight at 4°C in 5% bovine serum albumin in TBST containing either anti-p-JNK, anti-p-p38, or anti-p-IκBα (Cell Signaling Technology, Beverly, MA) at a dilution of 1:1000. 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

TABLE 1
Sequences of Primers Used for qRT-PCR Amplification

Target gene	GenBank accession no.	5′ → 3′ Primer sequences	Product length (bp)	T_m (°C)
Bcl-2	U34964	FW: ACTTTGCAGAGATGTCCAGT; RW: CGGTTCAGGTACTCAGCAT	217	55
PPAR-α	NM_001113418	FW: GATCGGCCTGGCCTTCTAAACATA; RW: TGCGTCGGACTCGGTCTTCTTGAT	205	60
PPAR-γ	NM_011146	FW: TCCGAAGAACCATCCGATTGAA; RW: GCAAGGCACTTCTGAAACCGACA	112	60
p38	NM_031020	FW: AGTGGCTGACCCTTATGAC; RW: CACAGTGAAGTGGGATGGA	168	55
IκBα	NM_001105720	FW: CTTGGTGACTTTGGGTGC; RW: ACTTCAACAGGAGCGAGA	113	55
p65	NM_199267	FW: ATGCGTTTCCGTTACAAG; RW: GTGAGGTGGGTCTTTGGT	156	55
JNK	XM_341399	FW: ATTTGGAGGAGCGAACTAAG; RW: ATTGACAGACGGCGAAGA	112	60
β-Actin	NM_031144	FW: TCGTGCGTGACATTAAAGAG; RW: ATTGCCGATAGTGATGACCT	134	57

Note. FW: forward primer; RW: reverse primer.

Western Blot Detection Kit (Tiangen, Beijing, China) and visualized by exposure to X-ray film (Kodak, NY). The intensities of the protein bands were quantified using a Tanon Imager program (Tanon, China). β-Actin expression was used to normalize for equal protein loading and to calculate the relative induction ratio.

Statistical analysis. Data were analyzed using SPSS for Windows 13.0 Software (SPSS, Inc., Chicago, IL) and presented as means with standard errors (mean ± SE). Differences between the control and the treatment groups were determined using a one-way ANOVA followed by the Duncan's multiple range test. A difference was considered significant at the conventional level of significance of $p < 0.05$.

RESULTS

Organ and Body Weight

Administration of 3 or 5 mg PFNA/kg/day to rats for fourteen days resulted in a significant decrease in body weight (18%, $p < 0.01$; and 39%, $p < 0.01$, respectively) compared with the control group (Fig. 1). At the end of the experiment, rats treated with 1 mg PFNA/kg/day exhibited a 24% increase in thymus weights ($p < 0.01$), whereas the 3 or 5 mg PFNA/kg/day treatment group displayed a 20% ($p < 0.01$) or 87% ($p < 0.01$) decrease in thymus weights, respectively (Fig. 1). Thymus weight relative to body weight was increased in 1 mg/kg/day group and decreased in 5 mg/kg/day group (Fig. 1).

Histopathology of the Thymus

Histological changes to the thymus were observed at the end of the second week of treatment. Thymi of the control rats exhibited a normal cortex/medulla ratio close to 2:1 (Fig. 2A). A few apoptotic lymphocytes were observed in the cortex of these rats (Fig. 2B). In PFNA-treated rats, increasing doses of PFNA lead to corresponding decrease in cortex:medulla ratio in the higher dose groups (Fig. 2C). The reduced cortex was associated with an increased number of cortical apoptotic lymphocytes and tingible body macrophages, which are macrophages that have ingested apoptotic cells (Fig. 2D).

Cytokines and Hormone

Alterations of the cytokine levels in the sera of the experimental rats were evaluated by ELISA. Rats dosed with

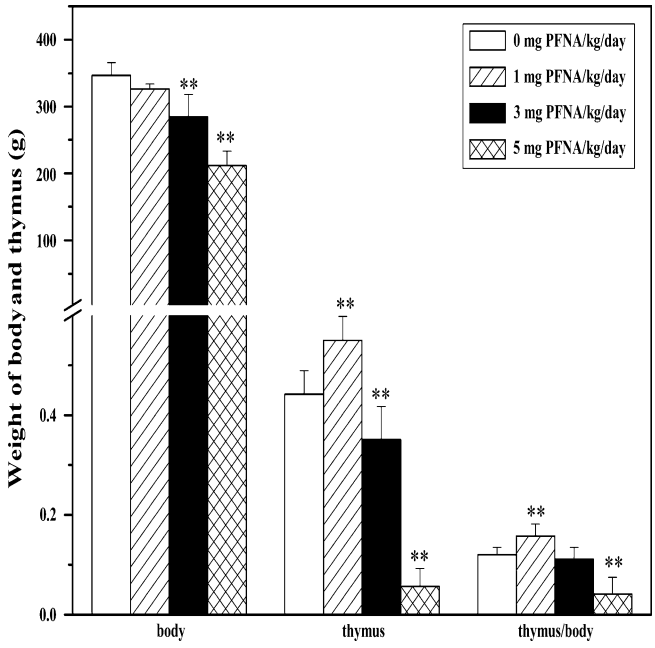

FIG. 1. Body weight and absolute and relative weight of thymi from control and PFNA-exposed male rats ($n = 6$). For all panels, the values are presented as the mean ± SE for six rats per group. $^{**}p < 0.01$.

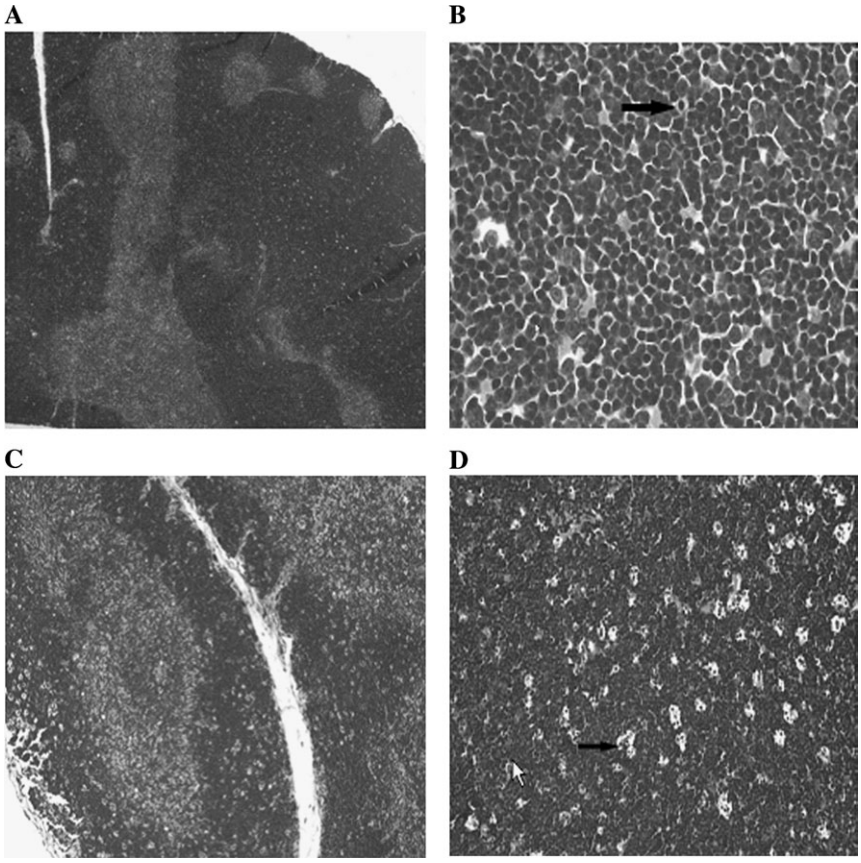

FIG. 2. (A) Low magnification of a normal thymus from a control rat exhibiting a cortex:medulla ratio of approximately 2:1 (×40). (B) High magnification of a normal thymus cortex from the same rat in (A) (×400). The arrow indicates an apoptotic body. (C and D) Thymus from a rat treated with 5 mg PFNA/kg/day (C) The thymus shows a cortex:medulla ratio of approximately 1:1 (×40). (D) Increased numbers of apoptotic lymphocytes and tingible body macrophages with intracytoplasmic apoptotic bodies (arrows) are evident in this thymus (×100).

3 or 5 mg PFNA/kg/day exhibited mean IL-1 concentrations that were increased 315% ($p < 0.01$) and 367% ($p < 0.01$), respectively, compared with the control group (Fig. 3A). The mean concentration of IL-4 in the highest dose group was 206% of the control group ($p < 0.01$), whereas no changes were observed in the other treated groups (Fig. 3A). On the contrary, the concentrations of IL-2 were significantly decreased by 29% and 40% in the 3 and 5 mg PFNA/kg/day groups, respectively ($p < 0.01$) (Fig. 3A).

RIA measurement was used to determine the cortisol concentration in sera samples. In rats receiving 5 mg PFNA/kg/day, an increased cortisol concentration, which was 167% ($p < 0.05$) of the control rats, was observed (Fig. 3B). No significant changes in cortisol levels were observed in the rats receiving the two lower doses of PFNA.

Gene Expression

Changes in expression levels of genes related to PPAR, MAPK, and NF-κB signaling pathways in the rat thymus were analyzed (Fig. 4). For 1, 3, and 5 mg PFNA/kg/day groups, the mRNA levels of PPAR-α were increased 2.2-fold, 4.6-fold, and 2.6-fold, respectively, compared with the controls ($p < 0.01$). In rats receiving 3 or 5 mg PFNA/kg/day, PPAR-γ expression levels were increased 2.9-fold and 1.7-fold, respectively, compared with the controls ($p < 0.01$). No differences in mean mRNA levels of JNK were observed between the control and PFNA-treated groups. Significant changes in p38 kinase expression occurred in the 3 and 5 mg PFNA/kg/day groups with a 397% increase in the 3 mg/kg/day group and a 70% decrease in the 5 mg/kg/day group ($p < 0.01$). No obvious changes were noted for expression of the NF-κB signaling genes p65 and IκBα following PFNA administration. In addition, the mRNA levels of the anti-apoptotic gene Bcl-2 were reduced 1.5-fold in the highest dose group ($p < 0.01$).

Western Blotting

In the thymi of rats receiving PFNA, p-JNK protein expression was 122, 129, and 124% of the control ($p < 0.01$) in the 1, 3, and 5 mg PFNA/kg/day groups, respectively (Fig. 5A). Increased p-p38 protein levels were observed in the 1 and 3 mg PFNA/kg/day treated groups (Fig. 5B); however, no differences in protein expression of p-IκBα was observed

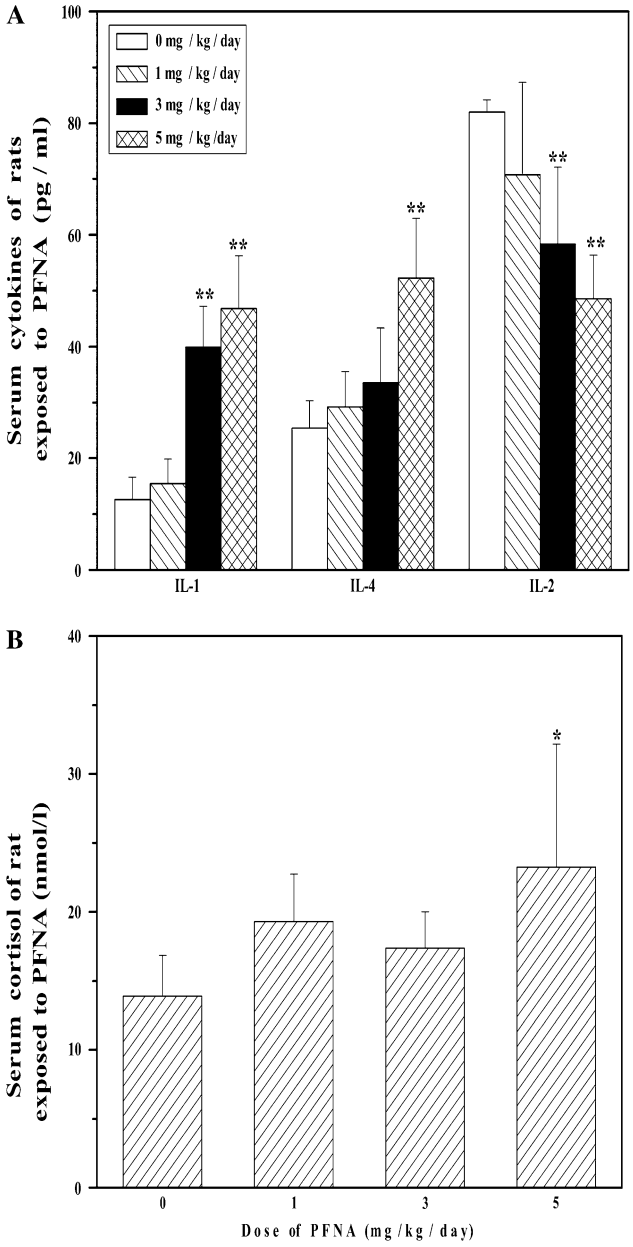

FIG. 3. Concentrations of cytokines (A) and cortisol (B) in the sera from control and PFNA-exposed male rats. Values are presented as the mean ± SE for six rats per group. $*p < 0.05$; $**p < 0.01$.

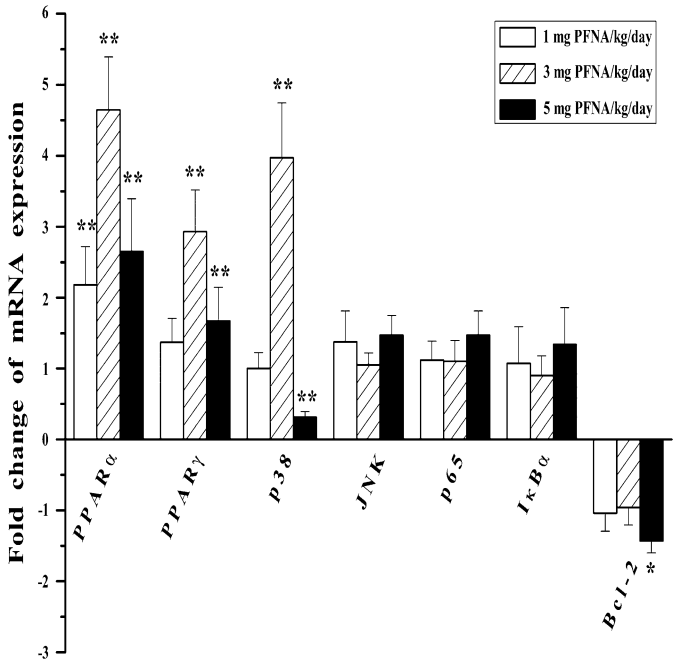

FIG. 4. Quantitative real-time PCR analysis of thymus mRNA expression levels of PPAR-α, PPAR-γ, p38, JNK, p65, IκBα, and Bcl-2 from control and PFNA-exposed male rats. Gene expression levels represent the relative mRNA expression compared with the control gene expression levels. Values indicate the mean ± SE for six rats per group. $*p < 0.05$; $**p < 0.01$.

between the PFNA-treated groups and the control group (Fig. 5C).

DISCUSSION

Previous studies have demonstrated an association between the immunotoxicity of PFAAs and the fluctuation of lymphoid cell subunits, suppression of lymphoid cell proliferation, and change of immunoglobulin production (Yang *et al*., 2002a, b). Due to the different methods, compounds, and experimental animals used, the results of these studies were not completely consistent. On the other hand, although these studies were exploring immunotoxicity of PFAAs, potential mechanisms were still intangible.

In the current study, we did not determine the PFNA concentration in serum, however, based on the data from De Silva *et al.* (2008), we extrapolate the concentration of PFNA in the serum of rats is approximate 10 μg/l at 1 mg/kg/day group exposed to PFNA for 14 days in this study, which are about 10 times of the mean PFNA concentrations in human serum (Calafat *et al*., 2006; Ericson *et al*., 2007) or five times of the higher mean concentrations found in a study of 20 U.S. citizens (Kuklenyik *et al*., 2004).

In the current study, there was an unexpected increase in thymus weight in the 1 mg PFNA group and the similar trends were observed in mice exposed to lower dose of PFOA (2.5, 6.25, and 12.5 mg/kg) (Fairley *et al*., 2007) and in rats administered with 0.3 mg APFO/kg/day for 29 days (Loveless *et al*., 2008). However, its biological significance for this increase in thymus weight is not clear. It is worthwhile to further investigate the immunomodulatory effects of the lower dose of PFAAs exposure in the future. Rats exposed to 3 or 5 mg PFNA/kg/day for fourteen days led to a remarkable decrease in body weight and lymphoid organ atrophy in male rats. This finding is in agreement with studies of PFOA on mice (Yang *et al*., 2002a, b) but different from studies of subchronic

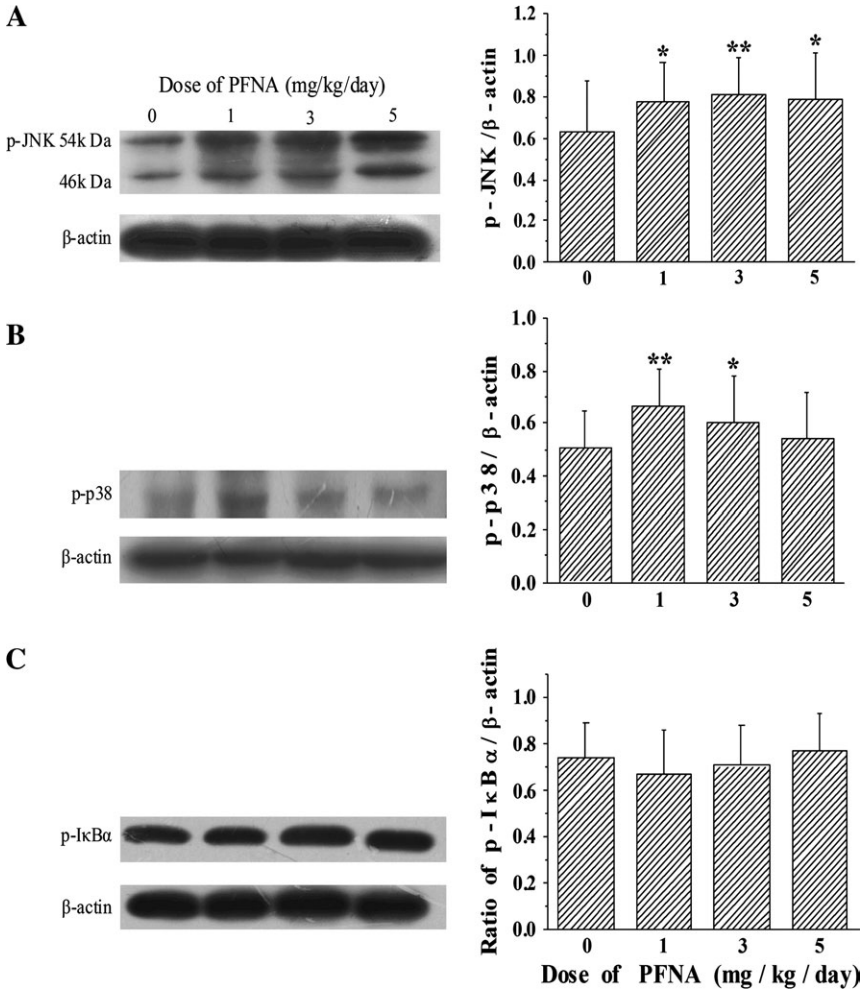

FIG. 5. Analysis of p-JNK, p-38, and p-IκBα protein expression. Representative western blots are shown on the left, and results from densitometry analysis of the western blots are shown in the right panels. These values are the means ± SE of three independent experiments with similar results. β-Actin was used as a loading control. $*p < 0.05$; $**p < 0.01$.

or chronic dietary exposure to PFOA in nonhuman primates or rodents (Butenhoff *et al.*, 2004; Griffith and Long, 1980). Previous studies have demonstrated the PFAAs-induced atrophy of rodents thymus attribute to the impairment of cell cycle and the dramatic decrease in cell number, especially the $CD4^{+}CD8^{+}$ T cell (Fang *et al.*, 2008; Yang *et al.*, 2000). In this study, the histopathological examination showed the obvious cell apoptosis in Sprague-Dawley rat thymus may be partly due to the reasons above.

The upregulated PPARs mRNA levels in the thymi of PFNA-treated rats in this study is quite similar to those in mice exposed to PFNA, indicating that PFNA is a PPAR agonist (Fang *et al.*, 2008). The upregulation of both PPAR-α and PPAR-γ showed PFNA had no selection for PPAR isotypes. Immunotoxicity studies of PFOA-treated transgenic PPAR-α null mice indicated the existence of PPAR-independent mechanisms and suggested an indirect mechanism of action such as cytokine production or lipid regulation (Yang *et al.*, 2002a). Recently, PFNA has been shown to affect the production of cytokines, IFN-γ, and IL-4 in spleen cells in mice (Fang *et al.*, 2008). Because serum cytokines are frequently used to assess immune function, this study examined the alteration of serum cytokines in PFNA-treated rats and demonstrated increased IL-1 and IL-4 levels and decreased IL-2 concentrations in the sera of the higher dose groups.

The physicochemical structure of the PFAA molecule suggests a possible interaction between the hydrophobic alkyl chain and serum proteins, and one of the possible sites of action may interact with serum proteins that are involved in endocrine and immunological functions (Jones *et al.*, 2003). These interactions may lead to immune or inflammatory responses. Loveless *et al.* demonstrated that exposure of mice to 10 or 30 mg APFO/kg/day challenged the immune system and increased the number of peripheral blood neutrophils and monocytes significantly (Loveless *et al.*, 2008). Because IL-1

is a prominent cytokine released by monocytes, neutrophils, and other macrophages during peripheral immune challenge (Ausiello *et al*., 1993), the increased serum IL-1 levels observed in this study may be due to possibly increased number of these cells in peripheral blood after PFNA exposure.

The marked increase in IL-1 will certainly activate the hypothalamic-pituitary-adrenal system, causing an elevation of systemic glucocorticoid (GC) levels (Chrousos, 1995; Johnson, 1997). In the present study, significantly elevated concentrations of cortisol, a type of GC, only occurred in the highest dose group, which may be due to the highest concentration of IL-1 that also occurred in this group. A similar increase in sera GC levels were observed in mice dosed with 10 or 30 mg APFO/kg/day, and in mice dosed with 5 mg PFNA/kg/day (Fang *et al*., 2008; Loveless *et al*., 2008). Compelling evidence has shown that GCs induce a shift from T helper1 (Th1) to T helper2 (Th2) immunity (Liberman *et al*., 2007). This shift was confirmed by a decrease in IL-2 levels, a hallmark characteristic of Th1 cells, and an increase in IL-4 levels, a signature cytokine of Th2 cells, in the highest dose group in this study. A decreased IL-2 concentration certainly suppresses lymphoid cell proliferation and thus affects the function of the immune system. IL-4 stimulates B cells to undergo immunoglobulin heavy chain class switching, and thus, this cytokine induces these cells to produce IgE and IgG1 rather than IgM and IgG2a (Agudelo *et al*., 2008; Takeda *et al*., 1996). Fairley *et al*. reported increased IgE in response to antigen in mice dermally treated with 25–50 mg PFOA/kg/day for four days. The authors postulated that PFOA-induced augmentation of IgE levels may be due to the increase in IL-4 production (Fairley *et al*., 2007), because other PPAR-α agonists, including gemfibrozil, ciprofibrate, and fenofibrate, have been shown to indirectly increase production of IL-4 (Lovett-Racke *et al*., 2004). On the other hand, several studies have demonstrated exposure to PFOA or PFOS resulted in suppression of the IgM antibody response to horse red blood cells or sheep red blood cells in rodents (Dewitt *et al*., 2008; Keil *et al*., 2008; Yang *et al*., 2002a). Increased serum concentrations of GC have been suggested to lead to inhibition of the IgM antibody response to antigen in mice treated with 10 or 30 mg APFO/kg body weight (Loveless *et al*., 2008). Thus, the remarkable increase in GC and IL-4 in the 5 mg PFNA/kg/day group in the current study may account for the different effects of PFAAs on the production of different immunoglobulins reported (Fairley *et al*., 2007; Loveless *et al*., 2008).

Increases of both cytokines and GCs trigger multiple signaling cascade pathways within target immune cells. Among these signaling pathways, MAPK signaling likely plays a more important role in the immune response. In mammals MAPKs include extracellular regulated kinase (ERK), JNK, and p38 kinase. JNK and p38 kinase are more sensitive to environmental stress, such as ultraviolet heat, osmotic shock, and inflammatory cytokines, and regulate many aspects of inflammation and immunity (Clark *et al*., 2003; Herlaar and Brown, 1999; Rincón *et al*., 2000). Increased IL-1 activates two MAPKs, JNK and p38 kinase (Dong *et al*., 2002). In addition, activation of PPAR has also been shown to activate JNK and p38 kinase (Gardner *et al*., 2003; Lennon *et al*., 2002). Thus, in the present study, increased IL-1 and upregulated PPARs suggested the possible activation of JNK and p38 kinases following PFNA treatment. Indeed, this notion was confirmed by the observed increase in protein levels of p-JNK and p-p38 in all PFNA-treated rats and the rats exposed to 1 or 3 mg PFNA/kg/day, respectively; however, no differences in JNK gene expression were noted between the control and treated rats. Alteration of p38 mRNA was vagarious as the levels were upregulated significantly in the 3 mg PFNA/kg/day group and downregulated significantly in the 5 mg PFNA/kg/day group. The loss of synchronization of the mRNA and protein levels of these kinases suggested the existence of post-transcriptional or post-translational modifications of these two proteins after PFNA exposure. The sudden decrease in p38 mRNA and p-p38 protein in the highest dose group may have stemmed from the increased levels of GCs, because GCs have previously been shown to inhibit the IL-1–induced phosphorylation and activation of p38 via upregulation of MAPK phosphatase-1 (Lasa *et al*., 2002).

Conflicting ideas about the role of MAPKs in cell death or survival after stress have been proposed (Hahn *et al*., 1992; Wilkinson and Kaye, 2001). Both p38 and JNK are pro-apoptotic, whereas ERKs are the modulators of cell survival after certain stimulations (Clanachan *et al*., 2003; Cicconi *et al*., 2003; Gao *et al*., 2002). Activation of JNK induces apoptosis by repressing the function of the anti-apoptotic proteins Bcl-2 and Bcl-xl (Kharbanda *et al*., 2000; Srivastava *et al*., 1999; Yamamoto *et al*., 1999). Furthermore, both JNK and p38 MAPK phosphorylate the Bcl-2 protein and attenuate its anti-apoptotic potential (Maundrell *et al*., 1997). This action may partly explain the thymocyte apoptosis observed in the rats in the lower PFNA dose groups in this study, which exhibit higher p-JNK and p-p38 protein levels. On the other hand, significant decline of Bcl-2 gene expression and the augmentation of GC levels may contribute to the severe thymocyte apoptosis observed in the highest dose group, because GCs are well known for induction of thymus apoptosis.

The NF-κB signaling pathway also plays an important role in regulating cell death by transcriptionally activating Bcl-2 (Chen *et al*., 2002). Both PPAR agonists and GCs are known to negatively regulate NF-κB signaling by upregulating the expression of IκBα and inhibiting the phosphorylation of IκBα in rodents (Almawi and Melemedjian, 2002; Dehmer *et al*., 2004); however, alterations in PPAR-α and PPAR-γ mRNA and GC levels did not affect the gene expression of IκBα kinase (IKK) and IκBα in PFNA-treated mice (Fang *et al*., 2008). In this study, exploration of the NF-κB signaling pathway in PFNA-exposed rats revealed that the mRNA levels of NF-κB p65 and IκBα and the protein level of p-IκBα were nearly consistent in all experimental rats. This result suggested

that the NF-κB signaling pathway may remain intact following PFNA exposure. This consistency may be the result of the counteracting functions of JNK and p38 kinases, because MAPK signal transduction pathways have been implicated in NF-κB activation through phosphorylation of its inhibitor IκBα (Castrillo *et al*., 2001; Lee *et al*., 1997; Schwenger *et al*., 1998). Due to the intricacies of the NF-κB signaling pathway response to various stimulants, additional research is warranted in order to determine its response during PFAA exposure.

In conclusion, following exposure of the rat immune system to PFNA, subsequent release of cytokines potentially influenced multiple facets of the immune system. These cytokines activated both p-JNK and p38 kinase, and the activation of these kinases affect anti-apoptotic Bcl-2 protein at the transcriptional and post-translational levels, leading to obvious cell apoptosis in the thymus. On the other hand, increased concentration of sera IL-1 stimulated GC production, which also contributed to the thymocyte apoptosis observed in the highest dose group. Although it was demonstrated in this study that the alterations of cytokines and MAPK signaling pathways were related to the immunotoxic effect of PFNA, further researches are necessary to explore the more detailed mode of actions affected by PFNA and how they eventually influence the immune system.

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