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MOLECULAR TOXICOLOGY

Kupffer cells suppress perfluorononanoic acid-induced hepatic peroxisome proliferator-activated receptor α expression by releasing cytokines

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Abstract Kupffer cells (KCs) have been demonstrated to play a role in the regulation of intra-hepatic lipid metabolism through the synthesis and secretion of biologically active products. The involvement of KCs in the disturbance of lipid metabolism that induced by perfluorononanoic acid (PFNA), a known agonist of the peroxisome proliferator-activated receptor alpha (PPARα), was investigated in this study. Rats were exposed to PFNA or PFNA combined with gadolinium chloride, an inhibitor of KCs, for 14 days. PFNA exposure dose-dependently increased absolute and relative liver weights, induced triglyceride accumulation, up-regulated the expression of both SERBP-1c and PPARα, and stimulated the release of TNFα and IL-1β. Inactivation of KCs markedly lowered TNFα and IL-1β level, enhanced PFNA-induced expression of PPARα and its target genes, and reduced liver triglyceride levels. In vitro, PFNA-induced expression of PPARα in primary cultured hepatocytes was suppressed by recombinant rat TNFα and IL-1β. However, inhibition of the NF-κB pathway prevented this. Transient transfection and promoter analysis further revealed that these two cytokines and NF-κB were coordinately involved in the suppression of PPARα promoter activity. Our data demonstrate that TNFα and IL-1β released from KCs following PFNA exposure can suppress the expression of PPARα via NF-κB pathway, which partially contribute to the evident accumulation of triglycerides in rat liver.

Keywords Peroxisome proliferator-activated receptors · NF-κB · Lipid metabolism · Hepatotoxicity · Perfluorinated compound

Abbreviations

PFC	Perfluorinated compound
PFNA	Perfluorononanoic acid
TG	Triglyceride
TCHO	Total cholesterol
LPS	Lipopolysaccharide
IL-1β	Interleukin-1 beta
IL-10	Interleukin-10
IL-6	Interleukin-6
TNFα	Tumor necrosis factor-alphaNF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells
PPARs	Peroxisome proliferator-activated receptors

Introduction

Kupffer cells (KCs) are the largest population of non-parenchymal cells in the liver (Bilzer et al. 2006). In

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addition to participating in homeostatic responses and immunomodulation, they are involved in hepatic metabolism through the synthesis and secretion of biologically active products, such as cytokines, reactive oxygen species, and chemokines (Kang et al. 2008; Odegaard et al. 2008).

Recently, KCs have been demonstrated to play pivotal roles in the pathogenesis of non-alcoholic fatty liver disease, the most common liver disease in Western countries (Marra et al. 2008; Rivera et al. 2007), because they are important sources of cytokines, such as TNFα and IL-1, which can induce lipogenesis in vitro in cultured hepatocytes and in vivo (Neyrinck et al. 2000; Graham and Brass 1994). A number of hepatotoxicants, including some PPARα agonists, are reportedly macrophage activators, and even at subtoxic doses, these compounds can dramatically affect liver function via KC activation (McMillian et al. 2004).

Perfluorononanoic acid (PFNA) is commonly used as emulsifier and surfactant in fluoropolymer manufacturing; it is one of the most frequently detected perfluorinated compounds (PFCs) in abiotic and biotic matrices (Hart et al. 2009; Calafat et al. 2007; Yeung et al. 2009) and has been reported to accumulate primarily in blood and liver (Giesy and Kannan 2001). Because the elimination half-life of PFCs in humans is several years, increasing concern has focused on their potential risk to human health (Lau et al. 2007).

It has been demonstrated that the serum concentrations of PFNA and other PFCs are positively associated with increased serum cholesterol in National Health and Nutrition Examination Survey participants in the USA (Nelson et al. 2010). Accumulation of these compounds in mammalian liver has been demonstrated to cause marked hepatotoxicity, such as hepatomegaly and peroxisome proliferation as well as alterations in enzymes involved in lipid metabolism and drug metabolism (Kudo and Kawashima 2003).

The effects of PFCs on lipid metabolism may be because they are weak agonists of peroxisome proliferator-activated receptors (PPARs), especially PPARα. Activation of PPARα and its target genes facilitate fatty acid oxidation and lowers lipid accumulation in the liver. However, liver triglyceride (TG) levels were markedly increased in rats after perfluorodecanoic acid administration (Zhang et al. 2008). Likewise, obvious hepatic lipid accumulation was observed in rodents after perfluorooctanoic acid (PFOA) treatment (Lau et al. 2007). Thus, other mechanisms may also be involved in the regulation of PFCs in liver lipid metabolism.

Kupffer cells have been reported to be involved in perfluorododecanoic acid-mediated transcriptional changes in hepatocytes via inflammatory or oxidative mediators (Maher et al. 2008). Additionally, activation of KCs exerts a negative effect on PFOA-induced peroxisomal enzyme activities (Youssef and Badr 1997).

However, to date, it remains unclear whether KCs are involved in PFC-induced hepatotoxicity, especially disorders of lipid metabolism. In this study, hepatic lipid metabolism was evaluated in rats exposed to PFNA or PFNA combined with gadolinium chloride ($GdCl_3$), a selective KC toxicant, to explore the role of KCs in the process. A series of in vitro experiments was also performed to examine the mechanism of the involvement of KCs in regulating hepatic lipid metabolism.

Materials and methods

Materials

Perfluorononanoic acid (CAS number: 375-95-1, 97 % purity), $GdCl_3$, lipopolysaccharide (LPS) from *Salmonella minnesota*, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant rat TNFα and IL-1β were obtained from PeproTech (Rocky Hill, NJ). The primary antibody for CD68 was purchased from Serotec (Düsseldorf, Germany); [^{13}C]2-PFNA (≥95 % purity) was purchased from Wellington Laboratories (Ontario, Canada).

Animal treatment

Male Sprague–Dawley rats (body weight, 120–130 g) were purchased from Weitong Lihua Experimentary Animal Central (Beijing, China) and kept under standard conditions. All experimental manipulations were performed in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals. After a 1-week acclimation period, 80 rats were divided into four groups of 20 and treated with 0, 0.2, 1, or 5 mg PFNA/kg/day by oral gavage for 14 days. During PFNA treatment, each of the four groups was further divided into two subgroups. One was injected intraperitoneally with $GdCl_3$ (10 mg/kg), dissolved in NaCl 0.9 %, and the other was injected with the same amount of NaCl 0.9 % twice per week. At the end of treatment, the rats were weighed, and six rats from each subgroup were euthanized by decapitation. Blood and liver were collected for further examination. The other rats from each subgroup were used for hepatocyte and KC collection.

Determining PFNA concentration in liver

Extraction of PFNA from liver samples was carried out using an ion-pairing method described previously (Taniyasu et al. 2003). The concentration of PFNA in livers was analyzed using high-performance liquid chromatography with tandem mass spectrometry (HPLC–MS/MS). A 10-μL aliquot of extract was injected onto a Keystone Betasil C18

column (2.1 mm i.d. × 50 mm length, 5 μm, 100 Å pore size, end-capped; Thermo Electron Corp.) with 2 mmol/L ammonium acetate and methanol as the mobile phases, starting at 10 % methanol and a flow rate of 300 μL/min. MS/MS parameters for the instrument were optimized for PFNA and have been reported previously (Taniyasu et al. 2005). The limit of quantification for PFNA was 10 ng/L.

Immunohistochemistry, serum biochemical parameters, and hepatic lipid analysis

To detect the effect of $GdCl_3$ on the activation of KCs, immunohistochemistry was adapted using primary antibody for CD68 according to Rivera et al. (2001). Serum biochemical parameters were measured using automated clinical laboratory tests. For hepatic TG and total cholesterol (TCHO) content quantification, samples were finely minced and extracted overnight in acetone at 4 °C. Extractable lipid content was measured using a commercially available kit (Biosino Bio-technology and Science Inc., Beijing, China). Levels of cytokines in 10 % liver homogenate were quantified using ELISA according to the manufacturer's protocol (Biosource International, Camarillo, CA).

Isolation of hepatocytes and KCs

Experimental rats were euthanized with pentobarbital sodium (50 mg/kg). Hepatocytes and KCs were isolated according to Smedsrød and Pertoft (1985). Cells were then washed three times with PBS before resuspension in TRIzol for RNA isolation.

Studies in primary culture hepatocytes and KCs

Hepatocytes or KCs were isolated from 200 ± 20 g rats as described previously (Smedsrød and Pertoft 1985). After overnight incubation, non-adherent cells were washed off, and the culture media replaced. The viability of cultures was >99 % (as assessed by trypan blue exclusion). The purity of KCs was >97 % [as assessed by CD68 immunofluorescent staining according to Babu et al. (2007)]. Hepatocytes were plated at a density of 1.5×10^6 cells/well in 6-well plates. All cells were cultured with complete medium (RPMI1640, 10 % fetal bovine serum, penicillin, streptomycin) and incubated at 37 °C in a 5 % CO_2 atmosphere. We performed experiments to detect the effect of PFNA on the viability of hepatocytes and KCs and found only higher doses of PFNA (≥100 μM) decreased their viability (The detail is given in the supporting information). Thus, the chosen in vitro treatments did not decrease cell viability. Cells were used in the following four experiments (The detail design was given in supporting information).

Rat PPARα promoter construct, transient transfection, and luciferase assay

The 5′-flanking −1,674/+117-bp region of the promoter is sufficient for full rat PPARα responsiveness. The complete promoter was synthesized using a PCR-based cloning strategy and subcloned into the pGL3 basic vector (Promega, Madison, WI), which contains the firefly luciferase reporter gene. The sense primer 5′-CGGGGTACCTGCAGGAGTTGGTTCTATCCTTCCACC-3′ and the antisense primer 5′-CCCAAGCTTCAGCGTCGCTTCAGTTCCAGGACT-3′ contain Kpn I and Hind III sites (underlined). The human p65 expression plasmid, cloned into a pFLAG-CMV2 vector, and the p-$[\kappa B]_3$-TK-Luc plasmid, containing three tandem repeats of the NF-κB-binding site, were gifts from Dr. Qinmiao Sun, Institute of Zoology, Chinese Academy of Sciences.

All transient transfections were performed using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The activity of luciferase was measured, and Renilla luciferase activities were determined to assess transfection efficiency. Assay results are expressed as relative luciferase activity.

Isolation of RNA and Real-Time PCR

Total RNA from hepatocytes and KCs was isolated by the TRIzol method according to manufacturer's instructions (Invitrogen). Reverse transcription was performed using an oligo-$(dT)_{15}$ primer and M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Real-time PCRs were performed using SYBR Green PCR Master Mix reagent kits (Takala, Dalian, China). Rat-specific primers were designed for genes of interest (Supplementary Information, Table S1).

Statistical analyses

Data were analyzed using the SPSS software (ver. 14.0 for Windows; SPSS, Inc., Chicago, IL) and are presented as means with standard errors (mean ± SE). Differences between the control and the treatment groups were determined using a one-way analysis of variance (ANOVA), followed by the Duncan's multiple range test. A difference was considered to be statistically significant at $P < 0.05$.

Results

KCs do not affect PFNA accumulation in liver

To investigate the importance of KCs in PFNA-induced hepatotoxicity, we performed liver KC inactivation using

gadolinium chloride ($GdCl_3$). As CD68 is a scavenger receptor present only on a subset of activated macrophages, the inactivation of KCs was evaluated by the quantification of CD68-positive cells by immunohistochemistry. CD68-positive cells were quantified in eight or nine fields of 10 sections using light microscopy. The results showed that the administration of $GdCl_3$ inactivated 95 % of the CD68-positive cells (Supplementary Information, Fig. S1).

Perfluorononanoic acid accumulated in rat liver in a dose-dependent manner after PFNA exposure for 14 days (Fig. 1), with increases in 79-fold and 880-fold up to 12.2 and 135.0 μg/g wet weight in the 0.2 mg and 5 mg/kg PFNA groups, respectively, compared with control group, suggesting that PFNA tended to accumulate in the liver. Inactivation of KCs using $GdCl_3$ did not affect the content of PFNA in rat liver after 14 consecutive doses.

Inactivation of KCs partially attenuates PFNA-induced hepatic toxicity

To examine the effects of KC inactivation on body weight and liver weight, rats were weighed at the beginning and the end of the experiment, and livers were weighed after decapitation. The results showed that only body weight in the 5 mg PFNA/kg-treated rats decreased 1.2-fold (Fig. 2a), whereas obvious hepatomegaly occurred and the absolute and relative liver weights increased in a dose-dependent manner by 1.7-foldand 2-fold in the 1 mg and 5 mg/kg PFNA groups, respectively (Fig. 2a), suggesting that PFNA exerted a greater effect on liver weight than on body weight. Inactivation of KCs by $GdCl_3$ had no significant effect on the PFNA-induced changes in body weight or relative liver weight, although the absolute liver weights were increased in the 1 mg/kg PFNA plus $GdCl_3$ group compared with the corresponding PFNA alone group ($P < 0.05$).

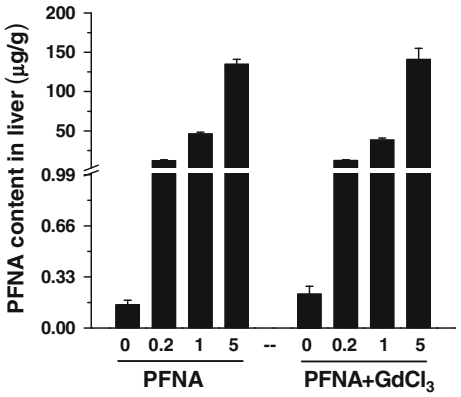

Fig. 1 PFNA content in rat liver. SD male rats were fed with PFNA for 14 days; half of the animals in each group were given an injection of 10 mg/kg $GdCl_3$ every other day. PFNA: 0.2, 1, and 5 mg/kg/d. Concentrations of PFNA in livers were analyzed using HPLC–MS/MS. *Error bars* indicate the standard error (SE, $n = 6$)

To examine the effects of KC inactivation on lipid storage, we measured lipid levels in liver and serum. TG levels in liver homogenate were increased significantly by 1.72-fold in the 5 mg PFNA/kg-treated group, compared with the control ($P < 0.05$). However, inactivation of KCs significantly decreased TG levels in the 5 mg PFNA/kg-dose group compared with the same dose of PFNA alone ($P < 0.01$). The TCHO level in liver showed no apparent change in any group (Fig. 2b). In serum, levels of TCHO and TG were dose-dependently decreased after PFNA treatment. Inhibition of KCs had little effect on PFNA-induced serum lipid changes (Fig. 2c).

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were increased, respectively, in 5 mg/kg only PFNA-exposed rats compared with the control. This result showed the occurrence of liver toxicity after PFNA exposure. However, inactivation of KCs partially counteracted this effect of PFNA and returned these enzymes almost to control levels except for AST activity (Fig. 2d).

KCs can suppress PPARα expression in liver

To further explore the role of KCs involved in TG storage induced by PFNA, we separated hepatocytes from PFNA-treated rats with and without KC inactivation using in situ perfusion, then measured the expression of a number of nuclear receptors and their key target genes involved in lipid homeostasis (β-oxidation: PPARα; lipogenesis: PPARγ, LXRα, and SERBP-1c). In PFNA groups without $GdCl_3$, genes related to fatty acid oxidation, such as PPARα and its target genes (Cpt1a, Acot1 and Acot2) in hepatocytes, were up-regulated significantly both at 1 mg/kg/d (except PPARα) and 5 mg/kg/d PFNA dose (Fig. 3a and b), whereas the expression of LXRα and PPARγ, which are involved in lipid storage, were unchanged (Fig. 3a). Additionally, SERBP-1c, which plays a major regulatory role in hepatic lipid biosynthesis, was induced significantly by 10-fold and 36-fold in 1 and 5 mg/kg/d PFNA group compared with the control, respectively (Fig. 3a).

Inactivation of KCs significantly enhanced expression of PPARα both at 1 and 5 mg/kg/d PFNA dose, and its target genes (Cpt1a and Acot1) at the highest dose compared with the corresponding group without KC inactivation. However, no effect of $GdCl_3$ was observed on the expression of genes involved in lipogenesis (PPARγ and SERBP-1c) (Fig. 3a). Taken together, these results suggest that inactivation of KCs enhanced PPARα-mediated fatty acid oxidation in hepatocytes.

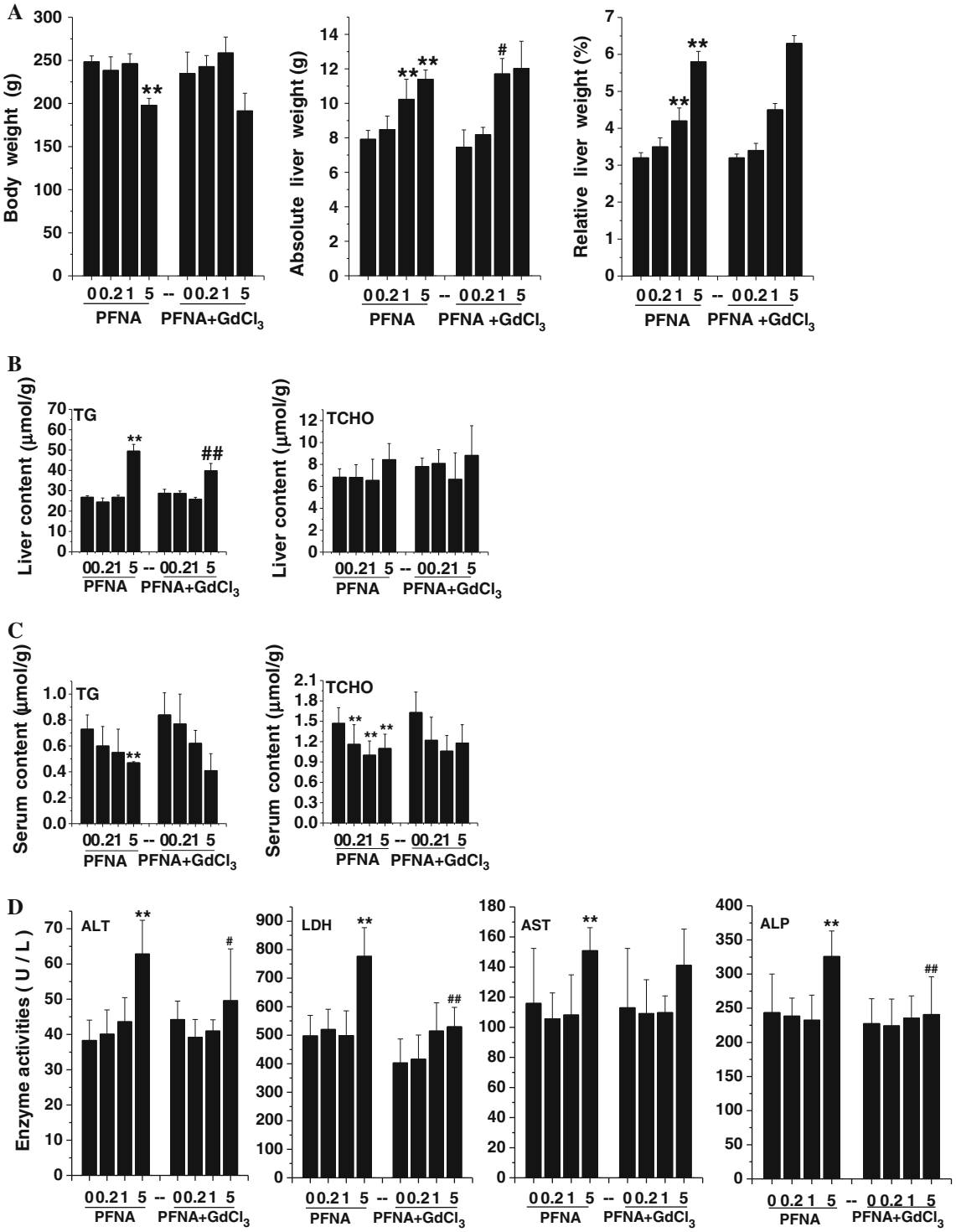

Fig. 2 Effect of Kupffer cell inactivation on weight, hepatic triglyceride content, and serum enzyme activities induced by PFNA. SD male rats were fed with PFNA for 14 days; half of the animals in each group were given an injection of 10 mg/kg $GdCl_3$ every other day. PFNA: 0.2, 1, and 5 mg/kg/d. **a** Body weight ($n = 10$), absolute liver weight ($n = 6$), and relative liver weight ($n = 6$). **b** Quantitative measurements of liver TG and TCHO ($n = 6$). **c** Levels of TG and TCHO in serum ($n = 6$). **d** Serum enzyme activity ($n = 6$). *Error bars* indicate SE. Differences were evaluated using a one-way analysis of variance (ANOVA), followed by the Duncan's multiple range test. **Significant difference from control, $P < 0.01$. $^{\#}$Significant difference from corresponding only PFNA-exposed group, $P < 0.05$, $^{\#\#}P < 0.01$

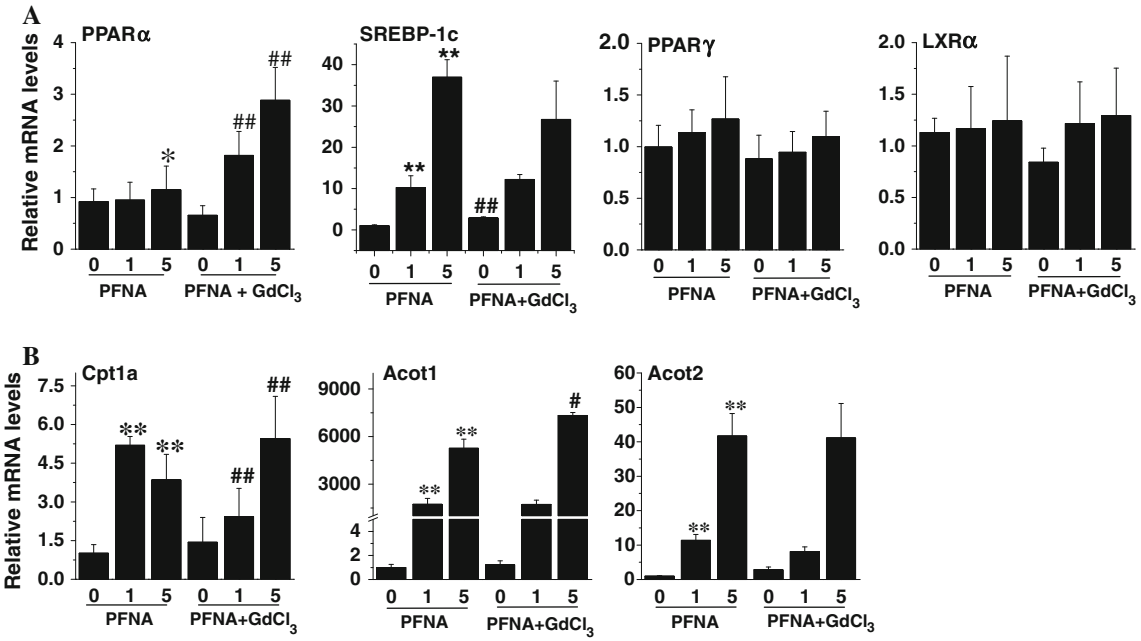

Fig. 3 The expression of nuclear receptors and their key target genes in vivo. SD male rats were fed with PFNA for 14 d, and half of the animals in each group were given an injection with 10 mg/kg $GdCl_3$ every other day. PFNA: 0, 1, and 5 mg/kg/d. **a** Expression of nuclear receptors in hepatocytes separated using in situ perfusion; **b** Expression of PPARα's target genes (Cpt1a, Acot1, and Acot2) in hepatocytes, as determined by qRT-PCR ($n = 4$). Differences were evaluated using one-way ANOVA, followed by the Duncan's multiple range test. *Significant difference from control, $P < 0.05$, **$P < 0.01$. #Significant difference from the corresponding only PFNA-exposed group, $P < 0.05$; ##$P < 0.01$

KCs were activated by PFNA and released TNFα and IL-1β

To address the potential mechanism underlying the effects of KCs on PPARα-dependent gene regulation in PFNA exposure, we next focused on factors derived from KCs. Levels of cytokines in rat liver homogenate after exposure to PFNA for 14 days were measured. Among them, TNFα and IL-1β were increased in a dose-dependent manner, with marked increases of 21-fold and 24-fold in the 5 mg/kg PFNA group ($P < 0.01$), respectively. IL-10 decreased dose-dependently with PFNA administration, and IL-6 levels decreased only in the liver of the 5 mg/kg PFNA group ($P < 0.05$). However, KC inactivation returned these cytokines to basal levels even with the highest PFNA exposure, indicating that TNFα and IL-1β in liver were specifically released from KCs stimulated by PFNA. On the other hand, inhibition of KCs had little effect on IL-6 levels, and IL-10 was elevated in the 5 mg PFNA/kg group compared with the same dose of PFNA alone (Fig. 4a).

To determine the effects of PFNA on the production of cytokines in KCs in vitro, KCs isolated from rat liver without PFNA treatment were exposed to PFNA for 24 h. We previously found that exposure of 0.2 and 5 mg/kg/d PFNA to mice for 14 days resulted in serum PFNA concentrations of 11.5 and 156.1 μg/mL (unpublished data). Five micromolars of PFNA is equal to 2.32 μg/mL, so these concentrations of PFNA are similar to those in serum following in vivo exposure. Levels of TNFα, IL-1β, IL-6, and IL-10 in the culture media were then determined. The results showed that 50 μM PFNA-augmented TNFα and IL-1β released from KCs by 2.2-fold and 3.7-fold, respectively, compared with the control. However, PFNA had no significant effect on KC-derived IL-6 or IL-10 (Fig. 4b).

TNFα and IL-1β inhibit the expression of PPAR via NF-κB pathway

We next examined the effects of TNFα and IL-1β on the expression of PPARα and its target genes in vitro. As seen in vivo, PFNA exposure induced expression of PPARα and its target genes in primary cultured hepatocytes isolated from rat liver without PFNA treatment, but co-exposure to TNFα or IL-1β with PFNA significantly suppressed the expression of these genes (Fig. 4c), whereas the expression of PPARγ and LXRα was unchanged (Supplementary Information, Fig. S2). These data suggest that cytokines, primarily TNFα and IL-1β, released from KCs, negatively interfere with PPARα action in rat primary hepatocytes, leading to reduced expression of PPARα target genes (Cpt1a, Acot1 and Acot2) in hepatocytes.

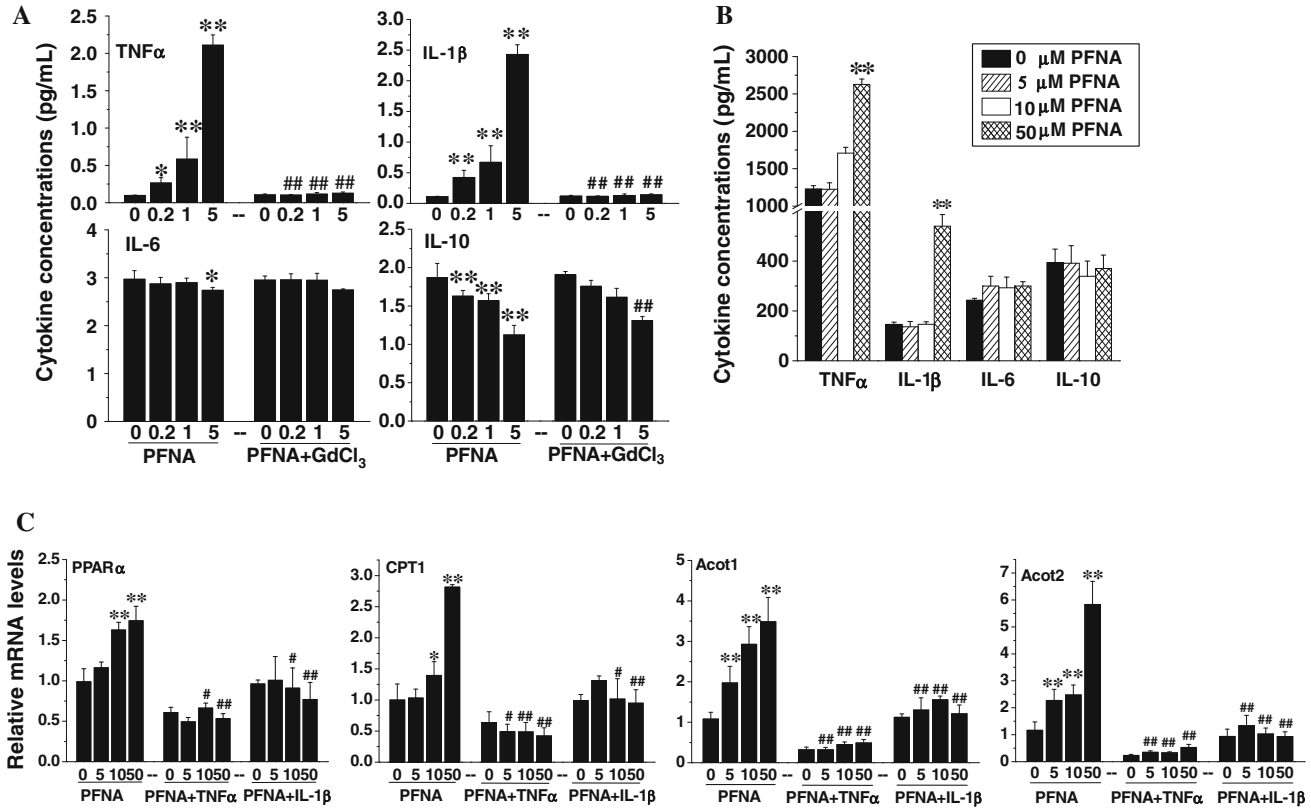

Fig. 4 Kupffer cells augment the release of cytokines after treatment with PFNA and reduce expression of PPARα and its target genes. SD male rats were fed with PFNA for 14 days; half of the animals in each group were given an injection of 10 mg/kg $GdCl_3$ every other day. PFNA: 0.2, 1, and 5 mg/kg/d. **a** Contents of TNFα, IL-1β, IL-6, and IL-10 in rat livers ($n = 6$). **b** Concentrations of TNFα, IL-1β, IL-6, and IL-10 in the culture media released by KCs isolated from naïve donors treated with PFNA ($n = 3$). **c** In vitro, primary hepatocytes isolated from naive donors were treated with PFNA or PFNA plus IL-1β (10 ng/mL) or PFNA plus TNFα (10 ng/mL) for 24 h ($n = 3$). PFNA: 0, 5, 10, and 50 μM, respectively. *Error bars* indicate SE. Differences were evaluated using one-way ANOVA, followed by the Duncan's multiple range test. *Significant difference from control, $P < 0.05$, $^{**}P < 0.01$. $^{\#}$Significant difference from the corresponding only PFNA-exposed group, $P < 0.05$, $^{\#\#}P < 0.01$

To investigate whether the NF-κB pathway was involved in this process, primary cultured hepatocytes isolated from rat liver without PFNA treatment were simultaneously treated with pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB. The results showed that PDTC reversed the suppressive effect of TNFα and IL-1β on PFNA-induced PPARα and its target genes expression (Fig. 5a and b), indicating that the NF-κB pathway is involved in the negative regulation of TNFα and IL-1β on PPARα and its target genes at the level of transcription.

TNFα and IL-1β inhibit PPAR promoter activity

To further assess which factor(s) activated NF-κB following PFNA exposure, HepG2 cells were transiently transfected with the NF-κB reporter p-κB3-TK-Luc at 24 h before administration of IL-1β, TNFα, IL-1β plus TNFα, PFNA, or WY-14,643. The luciferase assay showed that treatment with TNFα or IL-1β alone significantly increased the expression of the NF-κB reporter by 3.8-fold and 3.2-fold, respectively, and the combination of these two cytokines potentiated the effect by 4.4-fold. However, PFNA and WY-14,643 had little effect on the activation of NF-κB (Fig. 5c). These results suggest that TNFα and IL-1β are strong inducers of NF-κB activity.

Next, we studied the effect of the NF-κB p65 subunit on PPARα promoter activity. HepG2 cells were transiently transfected with the rat PPARα transcriptional reporter or co-transfected with a p65 plasmid and then treated with IL-1β and/or TNFα. The luciferase assay showed that treatment with IL-1β or TNFα apparently decreased rat PPARα promoter activity by 20 % ($P < 0.05$), and combined treatment with IL-1β and TNFα significantly suppressed promoter activity by 50 %. Notably, overexpression of p65 inhibited promoter activity by 90 %. Additionally, overexpression of p65 and treatment with both IL-1β and TNFα aggravated the suppressive effect on rat PPARα promoter activity (Fig. 6a). These data suggest that the inhibitory

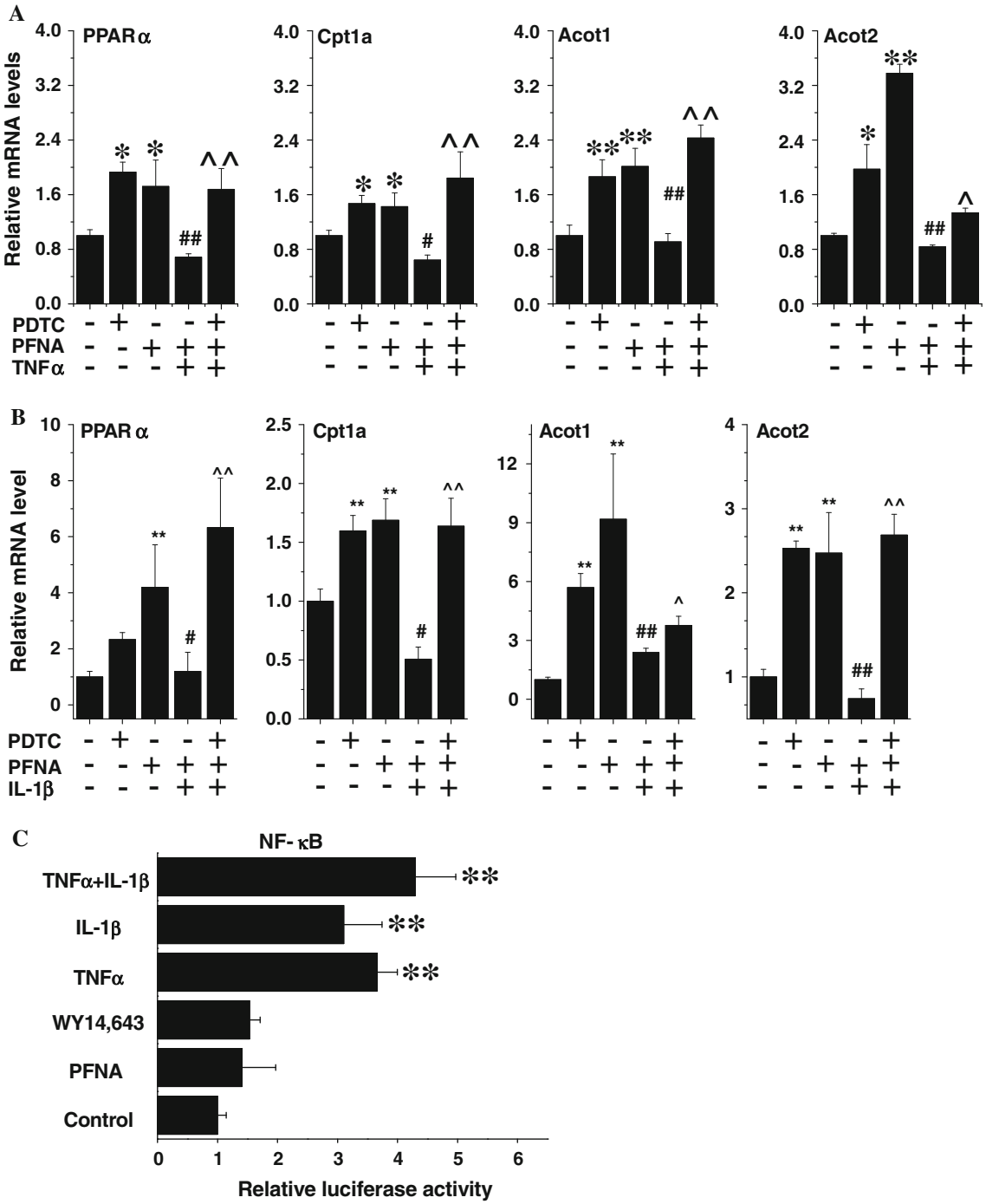

Fig. 5 TNFα and IL-1β inhibit the expression of PPARα and its target genes by activating NF-κB. The expression of PPARα and its target genes in primary hepatocyte isolated from naive donors was increased by treatment with PDTC, an inhibitor of NF-κB, for 24 h, as determined by qRT-PCR. **a** PFNA (50 μM), TNFα (10 ng/mL), PDTC (30 μM), $n = 3$. **b** PFNA (50 μM), IL-1β (10 ng/mL), PDTC (30 μM), $n = 3$. **c** TNFα and IL-1β activated the NF-κB reporter. HepG2 cells were transfected with the NF-κB reporter construct, followed by treatment with PFNA (50 μM), WY 14,643 (50 μM), TNFα, and IL-1β (10 ng/mL) for 36 h. *Error bars* indicate SE ($n = 3$). Differences were evaluated using Student's t test. *Significant difference from control, $P < 0.05$, **$P < 0.01$. #Significant difference from the corresponding only PFNA-exposed group, $P < 0.05$, ##$P < 0.01$. ^Significant difference from the group treated with PFNA and IL-1β/TNFα, $P < 0.05$, ^^$P < 0.01$

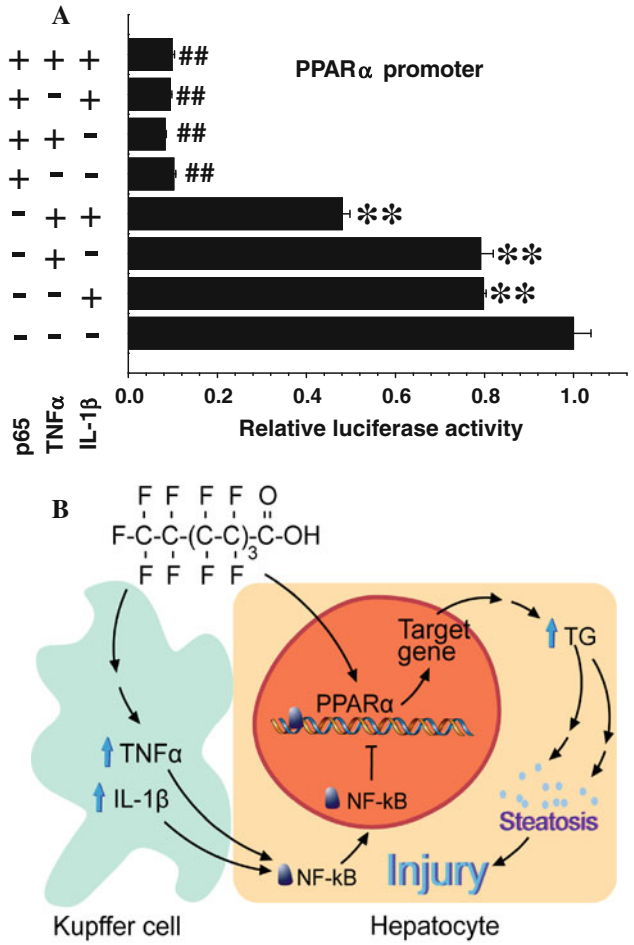

Fig. 6 TNFα and IL-1β suppress PPARα promoter activity via NF-κB. **a** HepG2 cells were transfected with rat PPARα promoter reporter constructs together with expression vectors for the NF-κB p65 subunit, followed by treatment with TNFα or IL-1β (10 ng/mL) for 36 h. Luciferase activity was determined in the cell lysates. *Error bars* indicate SE, $n = 3$. Differences were evaluated using Student's *t* test. **Significant difference from control, $P < 0.01$. ##Significant difference from the corresponding group without p65 transfection, $P < 0.01$. **b** Schematic model of KCs role in regulating lipid metabolism and hepatotoxicity

effects of those two cytokines on PPARα promoter activity are mediated by NF-κB.

Discussion

Accumulation of PFCs in the liver has been reported to interfere with hepatic lipid metabolism and to lead to lipid accumulation in hepatocytes via altering the expression of lipid metabolism-related genes (Lau et al. 2007). Our data showed PFNA exposure also increased liver lipid accumulation, concurrent with induced expression of both SERBP-1c and PPARα genes. It is interesting that the inactivation of KCs by $GdCl_3$ significantly up-regulated PPARα expression and reduced PFNA-induced hepatic TG storage, indicating KCs' important role in TG accumulation in rat liver exposed to PFNA.

Increasing evidence has demonstrated that KCs are involved in the regulation of lipid metabolism in adjacent hepatocytes. For example, inhibition of KC activity induces hepatic TG synthesis in fasted rats (Neyrinck et al. 2002), and depletion of KCs by $GdCl_3$ prevents the development of diet-induced hepatic TG accumulation and fatty acid esterification in rats (Huang et al. 2010). The effects of KCs on lipid metabolism have been demonstrated via released cytokines (Neyrinck et al. 2000; Graham and Brass 1994). For example, KC-derived IL-1β stimulates very low density lipoprotein–apolipoprotein B and lipid secretion in cultured hepatocytes (Bartolome et al. 2008). Inhibition of TNFα attenuates the detrimental effects of KCs on hepatocyte lipid metabolism (Huang et al. 2010). Treatment with IL-6 reduced hepatic lipid storage by increasing mitochondrial fatty acid oxidation and hepatic export of TGs and TCHO (Hong et al. 2004). The regulation of these cytokines in hepatocyte lipid metabolism may be related to the PPARα pathway. For example, PPARα expression was negatively regulated by Toll-like receptor-4 signaling and pro-inflammatory cytokines released by KCs (Rivera et al. 2007). Stienstra et al. (2010) demonstrated that treatment with IL-1β (intraperitoneal injection) decreased expression of PPARα and several of its target genes in mouse liver. In this study, exposure to PFNA in vivo or in vitro stimulated KCs to release large amounts of TNFα and IL-1β. Inactivation of KCs in PFNA-treated rats markedly decreased levels of IL-1β and TNFα in liver and increased the expression of PPARα and its target genes, suggesting the involvement of KC in hepatic lipid metabolism may occur via an interaction of these cytokines and the PPARα pathway. To further examine this, a series of in vitro experiment was performed. Our data showed that TNFα and IL-1β treatment suppressed expression of PPARα and its target genes, induced by PFNA in primary hepatocytes. However, pretreatment with an NF-κB inhibitor abolished this suppression, indicating the involvement of NF-κB in the suppression process. IL-1β and TNFα are known potent inducers of NF-κB, and peroxisome proliferators have also been reported to be able to activate NF-κB in KCs (Rusyn et al. 1998). However, in the present study, the luciferase assay showed that IL-1β or TNFα but not PFNA or WY-14,643, a potent peroxisome proliferator, markedly increased NF-κB activity in HepG2 cells, suggesting the activation role of peroxisome proliferator to NF-κB is indirect, and cytokines are involved in the process. Further, the results of the PPARα promoter experiment showed that overexpression of the NF-κB p65 subunit markedly decreased PPARα promoter activity, and intensified

suppression of the rat PPARα promoter by TNFα or IL-1β. Stienstra et al. (2010) also demonstrated that treatment with IL-1β, combined with overexpression of p50 and p65, markedly decreased human and mouse PPARα promoter activity in HepG2 cells compared with these factors alone. Thus, TNFα or IL-1β can suppress PPARα expression and activity via activation of the NF-κB pathway.

A growing body of evidence is consistent with up-regulation of PPARα protecting the liver against chemical-induced stress or damage (Mehendale 2000; Chen et al. 2000; Anderson et al. 2002). Minata et al. (2010) demonstrated that PPARα was protective against PFOA-induced hepatocyte and bile duct injuries and had an important role in drug-induced hepatobiliary injury. PPARα protects against obesity-induced chronic inflammation in the liver by direct down-regulation of inflammatory gene expression. In the present study, PFNA exposure for 14 days caused hepatotoxicity, revealed by serum enzymes (alanine aminotransferase, alkaline phosphatase, and lactate dehydrogenase); however, inactivation of KCs partially counteracted these effects of PFNA. Thus, suppression of PPARα activity by TNFα and IL-1β released from KCs may partially contribute to PFNA-induced hepatotoxicity.

In conclusion, PFNA exposure altered the expression of those genes related to lipid metabolism in vivo or in vitro and lead to lipid accumulation in the rat liver. Important cross talk between KCs and hepatocytes takes place in PFNA exposure. Activation of KCs by PFNA resulted in the release of TNFα along with IL-1β, causing suppressed expression of PPARα and its target genes by activating the NF-κB pathway. This further at least partially accelerates lipid accumulation in liver.

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