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Inflammation is independent of steatosis in a murine model of steatohepatitis

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Abstract

Obesity and alcohol consumption synergistically promote steatohepatitis, and neutrophil infiltration is believed to be associated with steatosis. However, the underlying mechanisms remain obscure. Peroxisome proliferator-activated receptor-gamma (PPAR γ) plays a complex role in lipid metabolism and inflammation, therefore, the purpose of this study was to dissect its role in regulating steatosis and neutrophil infiltration in a clinically relevant mouse steatohepatitis model of 3-month high-fat diet (HFD) feeding plus a binge of ethanol (HFD - plus-binge ethanol). Hepatocyte-specific *Pparg* disruption reduced liver steatosis, but surprisingly increased hepatic neutrophil infiltration after HFD-plus-binge ethanol. Knockout or knockdown of the PPAR γ target gene, fat-specific protein 27 (*Fsp27*), reduced steatosis without affecting neutrophil infiltration in this model. Moreover, hepatocyte-specific deletion of the *Pparg* gene but not the *Fsp27* gene markedly upregulated hepatic levels of *Cxcl1* (a chemokine for neutrophil infiltration) in HFD-plus-binge ethanol-fed mice. *In vitro*, deletion of the *Pparg* gene also highly augmented palmitic acid or TNF- α induction of *Cxcl1* in mouse hepatocytes. In contrast, activation of PPAR γ with a PPAR γ agonist attenuated *Cxcl1* expression in hepatocytes. Palmitic acid also upregulated IL-8 (a key chemokine for human neutrophil recruitment) expression in human hepatocytes, which was attenuated and enhanced by co-treatment with a PPAR γ agonist and antagonist, respectively. Finally, acute ethanol binge markedly attenuated HFD-induced hepatic PPAR γ activation, which contributed to the upregulation of hepatic *Cxcl1* expression post HFD-plus-binge ethanol. In conclusion, hepatic PPAR γ plays an opposing role in controlling steatosis and neutrophil infiltration, leading to dissociation between steatosis and inflammation. Acute ethanol gavage

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attenuates hepatic PPAR γ activation and subsequently upregulates hepatic CXCL1/IL-8 expression, thereby exacerbating hepatic neutrophil infiltration.

Keywords

PPARgamma; neutrophil; high fat diet; alcohol; CXCL1

Introduction

Excess alcohol consumption and obesity, renders increased risk for the development of steatohepatitis, cirrhosis and hepatocellular carcinoma.¹⁻⁴ The collaborative relationship between obesity and alcohol on steatohepatitis have been already observed and documented in clinical studies⁵⁻⁷ and in several animal models.⁸⁻¹⁰ However, the mechanisms underlying the interaction of fat and alcohol on steatohepatitis have not been extensively investigated due to lack of appropriate animal models that reproduce the full spectrum of human steatohepatitis.¹¹ For example, very little overlap of gene expression patterns was found between liver tissues from various mouse models of nonalcoholic fatty liver disease (NAFLD) and patients with different stages of NAFLD.¹² Among these various models, only high-fat diet (HFD) feeding associated hepatic gene expression change profiles were more similar to those in human fatty liver than other models.¹² HFD feeding for 3 months or longer resulted in severe steatosis and mild elevation of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) but limited inflammation with little neutrophil infiltration. Recently, we developed a mouse model of acute steatohepatitis (HFD-plus-binge ethanol model), in which administration of a single dose of ethanol after 3-month HFD feeding caused marked elevation of serum ALT and AST (reaching to approximately 800 IU/L) and significant hepatic neutrophil infiltration.¹³ This clinically relevant HFD-plus-binge ethanol model highlights the risk of even episodic binge drinking in obese/overweight individuals that binge drinking could cause significant hepatic neutrophil infiltration and acute steatohepatitis in these individuals. However, how binge drinking/acute ethanol gavage promotes hepatic neutrophil infiltration in HFD-fed mice or in obese individuals are not fully understood. For example, does steatosis promote liver inflammation through neutrophil infiltration?

Peroxisome proliferation-activated receptors (PPAR) have three isoforms (PPAR α , PPAR β/δ and PPAR γ), which belong to nuclear receptor superfamily of ligand-inducible transcription factors.¹⁴ The identified PPARs differ from each other in terms of tissue distribution and ligand specificity, acting as important regulators of many genes involved in adipogenesis, glucose homeostasis, lipid metabolism and inflammation.¹⁵ Given the therapeutic potential for type 2 diabetes, PPAR γ attracted extensive clinical interest and therefore has been extensively studied¹⁶ and tested in clinics for the treatment of NAFLD patients.¹⁷⁻¹⁹ However, the results from the treatment of NAFLD by PPAR γ agonists have been mixed,²⁰⁻²² which is partly due to the complex cell- and tissue-specific PPAR γ functions. PPAR γ is highly expressed in adipose tissues but at very low levels in normal liver.²³ Expression of PPAR γ is markedly upregulated in fatty livers in animal models^{24, 25} and in patients with NAFLD.²⁶ Disruption of the *Pparg* gene in hepatocytes ameliorates steatosis,

whereas overexpression promotes the development of fatty liver via the activation of various lipogenic genes, including fat-specific protein 27 (*Fsp27*),^{27, 28} and the resultant lipogenesis.^{23-25, 29} The *Fsp27* gene has two forms, *Fsp27a* and *Fsp27b*.³⁰ *Fsp27a* is highly expressed in white adipose tissues, whereas *Fsp27b* is highly expressed in brown adipose tissues and fatty livers.³⁰ FSP27 is a lipid droplet (LD) protein that plays an important role in LD formation and promotes the development of fatty liver diseases.^{27, 28, 31, 32} Although the steatogenic function of the PPAR γ -FSP27 axis is well documented, its role in hepatic neutrophil infiltration in steatohepatitis remains obscure.

Hepatic neutrophil infiltration is a hallmark of steatohepatitis and is believed to be associated with liver injury and disease progression via generating reactive oxygen species and producing pro-inflammatory mediators.³³ Our recent studies showed that blockade of several inflammatory mediators, such as chemokine (C-X-C motif) ligand 1 (CXCL1) and E-selectin, reduced hepatic neutrophil infiltration and ameliorated steatohepatitis in experimental animal model studies, confirming that neutrophil infiltration promoted hepatocyte injury.^{13, 34} Although the mechanisms underlying neutrophil infiltration in steatohepatitis remain obscure, it is generally believed that steatosis positively correlated with inflammation and liver injury. In the current study, we revealed that hepatic loss of PPAR γ ameliorated liver steatosis, but surprisingly aggravated hepatic neutrophil infiltration in mice treated with HFD-plus-binge ethanol. Mechanistically, we demonstrated that PPAR γ promoted steatosis via the upregulation of FSP27 and attenuated hepatic neutrophil infiltration via the downregulation of CXCL1/IL-8.

Materials and Methods

Mice

Mice were housed in a temperature-controlled room with a 12-h light/ 12-h dark cycle. C57BL/6J mice, Albumin-Cre mice, and *Pparg*^{fl/fl} mice on a C57BL/6J background were purchased from the Jackson Laboratory (Bar Harbor, ME). Hepatocyte-specific *Pparg* knockout (*Pparg*^{Hep-/-}) mice were generated through several steps of crossing Albumin-Cre mice and *Pparg*^{fllox/fllox} mice in the NIAAA animal facility. Hepatocyte-specific *Fsp27* knockout mice (*Fsp27*^{Hep-/-}) on a C57BL/6N background were generated as reported in our previous study and wild-type (WT) mice were used as controls.²⁷ All mice were cared for in accordance with the guidelines of the National Institutes of Health (NIH), and all animal experimental protocols were reviewed and approved by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) animal care and use committee.

HFD-plus-binge ethanol model

HFD-plus-binge ethanol model was previously described.¹³ Briefly, 8- to 12-week-old male mice were fed a HFD (60% kcal% fat; catalog no. D12492; Research Diet, New Brunswick, NJ) for 3 months (3m-HFD-fed mice). After 3m HFD feeding, mice received a single dose of ethanol (5 g/kg body weight as a 53.4% ethanol in water) via gavage at 7:00 AM, and were killed 9 hours later.¹³ The mice were not subjected to starvation before gavage. After gavage, the mice were kept on a heating pad (37°C) and the diet was removed.

Measurement of serum and liver free fatty acid (FFA) levels

Samples were prepared based on the manufacturer's instruction. Serum and liver FFAs were determined by using a BioVision kit (#K612-100; Milpitas, CA).

Measurement of serum, supernatant and liver CXCL1 protein levels

Samples were collected and prepared according to the manufacturer's instruction. Serum and liver CXCL1 levels, and CXCL1 levels in supernatant from cultured hepatocytes were measured by using an ELISA kit (#SMKC00B, R&D systems, Minneapolis, MN).

Adenovirus Fsp27 shRNA and adenovirus control shRNA

Generation and construction of adenovirus *Fsp27* shRNA (Ad-*shFsp27*) and adenovirus control shRNA (Ad-shRNA) were performed as described previously.²⁷ Briefly, 3m-HFD-fed mice were injected with 10^9 plaque formation units (Pfu) of Ad-shRNA or Ad-*shFsp27* in a total volume of 200 μ L PBS via tail vein injection, then the mice were subjected to binge ethanol administration 6 days later.

Statistical Analysis

In this study, all data are shown as the mean $\pm$ SEM (n=5-12 in each group). Group comparisons were performed using the unpaired t-test or one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons as appropriate. $P < 0.05$ was considered statistically significant.

Results

Hepatic *Pparg* deficiency improves hepatic steatosis but does not affect serum ALT and AST levels following HFD-plus-binge ethanol

Increased hepatic *Pparg* activation, a typical manifestation of fatty liver disease, promotes steatosis,²⁴ and it is generally believed that reduced hepatic steatosis is correlated with decreased serum ALT and AST levels.³⁵ Thus, we postulated that *Pparg*^{Hep-/-} mice had lower steatosis and liver injury than WT mice post HFD-plus-binge ethanol. As expected, compared to WT mice, *Pparg*^{Hep-/-} mice had milder liver steatosis and lower hepatic TG levels after HFD-plus-binge ethanol (Fig. 1A and 1B), but surprisingly serum levels of ALT and AST were not reduced in *Pparg*^{Hep-/-} mice (Fig. 1C). In addition, body weight gain and liver/body weight ratios were comparable between *Pparg*^{Hep-/-} mice and WT mice after HFD-plus-binge ethanol (Supporting Fig.1A and 1B).

Hepatic *Pparg* deficiency increases Cxcl1 expression and neutrophil infiltration following HFD-plus-binge ethanol

In our previous study, we demonstrated that acute inflammation played an important role in liver injury following HFD-plus-binge ethanol.¹³ Since less steatosis but similar liver injury was found in *Pparg*^{Hep-/-} mice compared with WT mice, we postulated that there was more inflammation in *Pparg*^{Hep-/-} mice. To test our hypothesis, liver inflammation was examined by performing neutrophil marker myeloperoxidase (MPO) staining. As illustrated in Fig. 2A and 2B, the number of MPO⁺ neutrophil was comparable in WT and *Pparg*^{Hep-/-} mice

following HFD feeding without ethanol binge. However, despite milder steatosis, *Pparg*^{Hep-/-} mice had a greater degree of hepatic neutrophil infiltration than WT mice after HFD-plus-binge ethanol, which was consistent with the finding that *Ly6g* (a marker for neutrophils) mRNA levels were higher in *Pparg*^{Hep-/-} mice (Fig. 2C). However, the number of Kupffer cells/macrophages as detected by F4/80 staining in the liver was similar between *Pparg*^{Hep-/-} mice and WT mice (Supporting Fig. 2A).

To understand the mechanisms by which *Pparg*^{Hep-/-} mice had greater hepatic neutrophil infiltration than WT mice after HFD-plus-binge ethanol, hepatic expressions of several chemokines were examined. In HFD feeding alone groups without binge ethanol, hepatic expression of *Cxcl1* mRNA level was approximately 5-fold higher in *Pparg*^{Hep-/-} mice than WT controls, while hepatic expression of other inflammatory markers, including *Cxcl2*, *Ly6g*, *IL-1β*, *IL-6* and *P-Selectin*, were comparable between WT and *Pparg*^{Hep-/-} mice (Fig. 2D). Compared to the HFD feeding alone groups, HFD-plus-binge ethanol highly upregulated hepatic expression of *Cxcl1* in both WT and *Pparg*^{Hep-/-} mice with much higher levels in *Pparg*^{Hep-/-} mice than in WT mice (Fig. 2D). HFD-plus-binge ethanol also upregulated hepatic expression of other inflammatory markers (such as *Cxcl2*, *E-selectin*, *P-selectin*) to a lesser extent, both in WT and *Pparg*^{Hep-/-} mice. Expressions of these inflammatory mediators were not higher in *Pparg*^{Hep-/-} mice compared to WT mice.

In addition, ELISA analyses confirmed that both hepatic and serum CXCL1 protein levels were much higher in *Pparg*^{Hep-/-} mice than WT mice following HFD-plus-binge ethanol (Fig. 2E, supporting Fig. 2B). Moreover, immunohistochemistry staining of CXCL1 also verified that there were more positive CXCL1-stained areas in *Pparg*^{Hep-/-} mice compared to WT controls following HFD-plus-binge ethanol (Fig. 2F).

The above data indicated that acute binge ethanol induced much higher levels of hepatic expression of *Ly6g* (neutrophil marker) and *Cxcl1* mRNAs in HFD-fed *Pparg*^{Hep-/-} mice than in HFD-fed WT mice. However, acute binge ethanol induced comparable levels of hepatic *Ly6g* and *Cxcl1* mRNA expression in chow-fed WT and *Pparg*^{Hep-/-} mice (supporting Fig. 2C). The reason why PPARγ has no effect on liver inflammation in chow-fed mice after acute ethanol binge is probably because chow-fed mice have very low hepatic PPARγ activation (lower nuclear PPARγ) in contrast to highly activated hepatic PPARγ in HFD-fed mice (reference,²⁴ also see results below in the current study).

Fsp27 is the downstream signal of Pparg-induced steatosis, but does not affect hepatic neutrophil infiltration in mice with HFD-plus-binge ethanol

The above data suggest that PPARγ promotes steatosis but inhibits neutrophil infiltration-induced inflammation. To understand the underlying mechanisms, we examined hepatic expression of *Fsp27*, a downstream target gene of PPARγ,²⁸ to see whether *Fsp27* mediates both the steatogenic and anti-inflammatory effect of PPARγ. *Fsp27* exists two isoforms of *Fsp27* gene, including *Fsp27a* and *Fsp27b*.³⁰ In our previous study, *Fsp27* was reported to be highly up-regulated in mice on chronic-plus-binge ethanol model, and up-regulation of *Fsp27* contributed to alcohol-induced liver injury.²⁷ Here we found that HFD-plus-binge ethanol induced much higher levels of hepatic *Fsp27a* and *Fsp27b* mRNA in mice than HFD feeding alone, and that both *Fsp27a* and *Fsp27b* mRNA levels were markedly reduced in

Pparg^{Hep-/-} mice compared to WT mice after HFD feeding or HFD-plus-binge ethanol (Fig. 3A). In addition, mRNA encoding hepatic *Cidea* were also reduced, but mRNA encoding hepatic *Cideb* were not reduced in *Pparg*^{Hep-/-} mice compared with WT mice (Fig. 3A).

In order to test the pro-steatotic function of FSP27, *Fsp27shRNA* adenovirus was used to knock down *Fsp27* gene expression. There were no changes in HFD consumption and body weight in mice 3 days or 6 days after injecting with control *shRNA* and *Fsp27shRNA* adenovirus (Supporting Fig. 3A). Both hepatic *Fsp27a* and *Fsp27b* mRNAs were lowered by *Fsp27shRNA* adenovirus injection, thus confirming hepatic *Fsp27* expression was reduced (Supporting Fig. 3B).

In both the HFD feeding alone and HFD-plus-binge ethanol groups, injection of *Fsp27shRNA* adenovirus reduced liver steatosis (Fig. 3B) and ameliorated hepatic TG levels (Supporting Fig. 3C). Interestingly, compared to the *shRNA* control, injection of *Fsp27shRNA* reduced serum ALT and AST levels in HFD-plus-binge ethanol groups but not in HFD feeding alone groups (Fig. 3C).

Furthermore, genetic disruption of *Fsp27* did not affect body weight (Supporting Fig. 3D) but led to reduced steatosis in both HFD and HFD-plus-binge ethanol groups (Fig. 3D). Both serum ALT and AST levels were markedly reduced in *Fsp27*^{Hep-/-} mice compared to WT mice in HFD-plus-binge ethanol groups, whereas only ALT levels were reduced in *Fsp27*^{Hep-/-} mice in HFD alone groups (Fig. 3E). Thus, *Fsp27* is responsible for the pro-steatotic function of hepatic PPAR γ in this HFD-plus-binge ethanol model.

In order to investigate the anti-inflammatory function of *Fsp27* in HFD-plus-binge ethanol model, we tested neutrophil infiltration by performing MPO staining and real-time PCR analysis of *Ly6g* mRNA in mice with hepatic *Fsp27* knockdown and *Fsp27* disruption. As illustrated in Fig. 4A-D, neither *Fsp27* knockdown nor *Fsp27* deletion altered the number of infiltrated neutrophil and hepatic expression of *Ly6g* in HFD feeding alone or in HFD-plus-binge ethanol groups, except for a mild increase in the number of infiltrated neutrophil following *Fsp27* knockdown in HFD feeding alone. Moreover, hepatic expression of *Cxcl1* mRNA was not altered by hepatic *Fsp27* knockdown or *Fsp27* disruption (Fig. 4E).

The above data suggest that FSP27 does not play a role in inducing hepatic neutrophil infiltration and *Cxcl1* mRNA expression in HFD-fed mice even when hepatic *Fsp27* mRNA expression is highly elevated. Because FSP27 is expressed at very low levels in the livers from chow-fed mice,²⁷ FSP27 very unlikely affects hepatic *Cxcl1* mRNA expression and neutrophil infiltration in these mice.

Hepatic Pparg deficiency reduces liver fibrogenic response/fibrosis in HFD-plus-binge ethanol

Liver steatosis and inflammation are associated with the development of fibrosis.³⁶⁻³⁹ Previous studies reported an anti-inflammatory and anti-fibrogenic properties of PPAR γ in non-parenchymal liver cells.⁴⁰ Thus, we wondered whether hepatic PPAR γ also affected liver fibrogenic response/fibrosis in post HFD-plus-binge ethanol. Compared to WT mice, *Pparg*^{Hep-/-} mice had fewer areas of Sirius red staining following HFD feeding or HFD-plus-

binge ethanol (Fig. 5A-C). In addition, *Tgfb*, *Col1a1*, *Col3a1*, *Col4a1* and *Col12a1* mRNAs encoding fibrogenic markers, were reduced in *Pparg*^{Hep-/-} mice compared to WT mice post HFD-plus-binge ethanol.

As PPAR γ in hepatocyte oppositely regulates steatosis and inflammation as demonstrated above, it remains a question whether steatosis dominantly modulates fibrosis instead of inflammation in the HFD-plus-binge ethanol model. To answer this question, we further tested liver fibrosis by performing hepatic *Fsp27* knockdown and *Fsp27* knockout. As shown in Supporting Fig. 4A-B, Sirius red staining revealed that less fibrosis lesions were found in the *Fsp27* knockdown and the *Fsp27* knockout groups, after both HFD feeding and HFD-plus-binge ethanol challenge. Because knockdown/knockout of *Fsp27* reduced steatosis but not inflammation, the data from Supporting Fig. 4 suggest that steatosis not inflammation plays a predominate role in promoting liver fibrosis in this HFD-plus-binge ethanol model.

Interaction of PPAR γ and NF- κ B regulates *Cxcl1* expression in hepatocytes

To understand the mechanisms by which activation of PPAR γ inhibits the upregulation of *Cxcl1* in the liver, we isolated primary hepatocytes and treated them with different regimens of palmitic acid (PA) and/or troglitazone (a PPAR γ agonist). Treatment with PA markedly upregulated *Cxcl1* mRNA expression in hepatocytes (Fig. 6A). Interestingly, PA-mediated upregulation of *Cxcl1* mRNA was markedly enhanced in hepatocytes from *Pparg*^{Hep-/-} mice (12-fold induction in *Pparg* knockout hepatocytes vs. a 2-fold change in WT hepatocytes). In addition, treatment with TNF- α also upregulated *Cxcl1* mRNA expression in isolated hepatocytes with much higher levels in *Pparg* knockout hepatocytes than WT hepatocytes (Fig. 6B). In contrast, activation of PPAR γ by treatment with troglitazone attenuated TNF- α -mediated upregulation of *Cxcl1* mRNA and CXCL1 protein in hepatocytes (Fig. 6C). Surprisingly, treatment with troglitazone did not affect PA-mediated upregulation of *Cxcl1* mRNA in hepatocytes (Supporting Fig. 5). Moreover, we have previously demonstrated that NF- κ B activation is involved in upregulation of *Cxcl1* mRNA in WT hepatocytes.¹³ Here we found that treatment with the NF- κ B inhibitor Bay also markedly reduced PA-mediated induction of *Cxcl1* mRNA in *Pparg* knockout hepatocytes (Fig. 6D).

Finally, IL-8 is a functional homologue of mouse *Cxcl1* and its expression in human hepatocytes is upregulated by PAs,⁴¹ playing a critical role in promoting neutrophil infiltration in human steatohepatitis. Thus, we determined whether PPAR γ also regulates IL-8 expression in human hepatoma HepG2 cells. Treatment with PA induced *IL8* mRNA in HepG2 cells. Co-treatment with the PPAR γ agonist troglitazone inhibited PA-mediated upregulation of *CXCL1* mRNA, whereas co-treatment with the PPAR γ antagonist GW9662 enhanced it (Fig. 6E).

HFD feeding activates hepatic PPAR γ , which is attenuated by a binge of ethanol

Several murine models of obesity and diabetes developed fatty liver with enhanced levels of PPAR γ expression.⁴² Previous studies also showed that *Pparg* mRNA level was downregulated in response to the combination of acute ethanol and lipopolysaccharide (LPS).⁴³ To further clarify the modulation of hepatic PPAR γ in response to HFD-plus-binge ethanol, we examined nuclear PPAR γ staining in this model. Very few PPAR γ positive

nuclei of hepatocytes were detected in chow- and chow-plus-binge ethanol-fed mice (Fig. 7A). However, a significant number of PPAR γ -stained nuclei of hepatocytes were found in mouse livers from HFD and HFD-plus-binge ethanol with a lower number in the HFD-plus-binge ethanol group compared to HFD feeding group. Western blot analyses confirmed that expression of nuclear PPAR γ protein (activated PPAR γ) in hepatocytes was increased by 12 folds in HFD-fed mice as compared with chow-fed mice, but was significantly suppressed in the HFD-plus-binge ethanol-fed mice compared to that in HFD alone-fed mice (Fig. 7B-C). In addition, hepatic expression of total PPAR γ protein was also higher in HFD-fed mice than in chow-fed mice, and that acute ethanol binge markedly downregulated hepatic expression of PPAR γ protein in HFD-fed mice (Fig. 7B-C). Moreover, hepatic expression of *Pparg* mRNA was much higher in HFD-fed mice than in chow-fed mice, and such expression was markedly attenuated in mice following HFD-plus-binge ethanol (Fig. 7D).

Discussion

By using a clinically relevant mouse steatohepatitis model induced by HFD-plus-binge ethanol, mechanistic insights into the role of steatosis and inflammation in liver disease were investigated leading to several novel findings. First, hepatic PPAR γ promoted steatosis via *Fsp27* upregulation while attenuating neutrophil infiltration via CXCL1 suppression. Second, acute ethanol binge inhibits PPAR γ activation, which is partially responsible for acute ethanol induced upregulation of hepatic CXCL1. Third, acute ethanol gavage induces hepatic neutrophil infiltration in HFD-fed mice independent of steatosis. Finally, PPAR γ agonists also inhibit IL-8 expression in human hepatocytes. These findings were used to generate a working model depicting the interaction of fat and acute ethanol in promoting steatohepatitis (Fig. 8).

The role of hepatic PPAR γ in the development of fatty liver has been well documented in several animal models of fatty liver induced by HFD feeding,²⁴ chronic ethanol feeding,²⁵ or genetic deletion of leptin.²³ Given all of these models are associated with little liver inflammation (eg. neutrophil infiltration), the effect of hepatic PPAR γ in liver inflammation has not been investigated and still remains obscure. In the current study, we used a recently developed mouse model of HFD-plus-binge ethanol, which induces significant hepatic neutrophil infiltration,¹³ to study the role of PPAR γ in modulating steatosis and neutrophil recruitment in the liver. The most striking finding was that although steatosis was reduced, hepatic neutrophil infiltration was markedly exacerbated in *Pparg*^{Hep-/-} mice after HFD-plus-binge ethanol, suggesting that hepatic PPAR γ acts an anti-inflammatory pathway via the inhibition of hepatic neutrophil infiltration. We have previously demonstrated that hepatic CXCL1, a key chemokine to promote neutrophil infiltration, was upregulated by more than 30-fold after HFD-plus-binge ethanol, and played an important role in hepatic neutrophil infiltration in this model.¹³ Fascinatingly, in the current study, we found that hepatic and serum levels of CXCL1 protein were further elevated in *Pparg*^{Hep-/-} mice compared to those in WT mice (approximately 2500 pg/ml in serum from *Pparg*^{Hep-/-} mice vs 1200 pg/ml in WT) after HFD-plus-binge ethanol, indicating that hepatic PPAR γ played a critical role in inhibiting CXCL1 protein production in hepatocytes, thereby limiting hepatic neutrophil infiltration. The inhibitory effect of PPAR γ on *Cxcl1* mRNA expression in hepatocytes were also confirmed in cultured primary hepatocytes, as disruption of the *Pparg* gene in

hepatocytes upregulated FA- or TNF- α -induced *Cxcl1* mRNA, while activation of PPAR γ by treatment with the PPAR γ agonist troglitazone decreased TNF- α -induced *Cxcl1* mRNA levels. Interestingly, activation of PPAR γ by troglitazone did not affect PA-mediated *Cxcl1* mRNA expression in hepatocytes. The reason why troglitazone treatment does not affect PA-mediated upregulation of hepatic *Cxcl1* mRNA is probably because PA itself is a strong ligand for PPAR γ activation⁴⁴ and thus may diminish the effect of PPAR γ agonist treatment.

Mechanistically, we also demonstrated that inhibition of NF- κ B abolished PA-mediated upregulation of *Cxcl1* mRNA in *Pparg*^{Hep-/-} hepatocytes, suggesting that PPAR γ attenuates hepatic *Cxcl1* mRNA levels via an NF- κ B-dependent mechanism. Several previous studies suggested that PPAR γ inhibited the expression of pro-inflammatory genes by interfering with NF- κ B DNA binding.⁴⁵⁻⁴⁹ This mechanism likely also explains the PPAR γ inhibition of *Cxcl1* gene expression in hepatocytes via the inhibition of FFA activation of NF- κ B. In addition, hepatic and serum FFA levels were comparable in *Pparg*^{Hep-/-} mice compared to WT mice following HFD-plus-binge ethanol (Supporting Fig. 6A-B). Taken together, these data suggest that upregulated hepatic *Cxcl1* mRNA expression in *Pparg*^{Hep-/-} mice is due to enhanced FFA signaling (through activation of NF- κ B) in hepatocytes, and is not due to altered FFA levels compared to WT mice.

Liver fibrosis is always associated with steatosis and inflammation. Hepatic steatosis generally promotes liver fibrogenesis by producing reactive oxygen species and danger associated molecule patterns (DAMPs), while inflammation plays a complex role in promoting or suppressing liver fibrogenesis.^{37, 38} Interestingly, compared to WT mice, *Pparg*^{Hep-/-} mice had reduced liver steatosis and fibrosis although these mice had enhanced liver inflammation post HFD-plus-binge ethanol. These results suggest that hepatic PPAR γ promotes liver fibrosis via the stimulation of hepatic steatosis, while the anti-inflammatory effects of hepatic PPAR γ are less important for liver fibrosis in the HFD-plus-binge ethanol model.

Hepatic PPAR γ was upregulated and activated in mice following HFD feeding^{24, 25} or chronic ethanol feeding.²⁵ Here we found that hepatic PPAR γ was also highly upregulated and activated after HFD feeding, but surprisingly, this upregulation and activation were attenuated by an additional ethanol binge challenge. As a result, the attenuation of hepatic PPAR γ likely diminished the inhibitory effect of PPAR γ on hepatic neutrophil infiltration, resulting in highly elevated hepatic CXCL1 level and hepatic neutrophil infiltration observed in HFD-plus-binge ethanol-fed mice compared to HFD alone-fed mice. In addition, HFD feeding or acute ethanol gavage alone induced fat accumulation in the liver, but HFD-plus-binge ethanol did not further exacerbate steatosis compared to HFD feeding alone.¹³ One possible explanation with regard to underlying mechanisms may be that in addition to promote steatosis, acute ethanol binge also reduced the steatogenic effect of PPAR γ via the inhibition of PPAR γ activation, resulting in comparable levels of steatosis in HFD-plus-binge ethanol and HFD feeding groups. Another interesting observation was that acute ethanol binge downregulated hepatic expression of PPAR γ protein and mRNA in HFD-fed mice as demonstrated in the current study, but chronic ethanol feeding upregulated hepatic *Pparg* expression as reported in a previous study.²⁵ However, the mechanisms by which

hepatic PPAR γ is oppositely regulated by acute and chronic ethanol feeding remain unknown, and further studies are required to clarify.

Hepatic neutrophil infiltration, a hallmark of steatohepatitis, is generally believed to be associated with the degree of steatosis.³³ In the current study, we provided evidence suggesting that hepatic neutrophil infiltration is independent of steatosis in HFD-plus-binge ethanol model. First, *Pparg*^{Hep-/-} mice had significantly reduced steatosis but had increased hepatic neutrophil infiltration than WT mice post HFD-plus-binge ethanol. Second, inhibition of the PPAR γ downstream target gene *Fsp27* also markedly improved steatosis but did not affect hepatic neutrophil infiltration and hepatic *Cxcl1* mRNA expression in mice fed with HFD-plus-binge ethanol.

Another important finding from the current study is that activation of PPAR γ not only inhibits PA-mediated induction of CXCL1 in mouse hepatocytes but also suppresses PA stimulation of IL-8, a functional homologue of mouse CXCL1, in human hepatocytes. PA induction of IL-8 in human hepatocytes likely plays an important role in promoting neutrophil infiltration and disease progression in human nonalcoholic steatohepatitis (NASH).⁴¹ Thus, treatment with PPAR γ agonists may be beneficial for human NASH via the inhibition of IL-8 production in hepatocytes.

Clinical implications of this study

The complex effects of PPAR γ on hepatic steatosis, fibrosis, and neutrophil infiltration compromise the potential use of PPAR γ as a therapeutic target for the treatment of NASH.¹⁹ The PPAR γ agonist pioglitazone, a prescription drug for type 2 diabetes, should be cautiously used for the treatment of NASH because activation of hepatic PPAR γ while beneficial for the inhibition of neutrophil infiltration, could exacerbate steatosis and fibrosis. Furthermore, the PPAR γ -NF- κ B-CXCL1/IL-8 axis identified in the current study yields new insights into the development of potential treatment of neutrophil-mediated steatohepatitis reflecting the synergistic effect of obesity and alcohol consumption in hepatic neutrophil infiltration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AH

alcoholic hepatitis

ALT

alanine aminotransferase

AST

aspartate aminotransferase

CXCL1

(C-X-C motif) ligand 1

FFAs

free fatty acids

FSP27

fat-specific protein 27

HFD

high-fat diet

HFD-plus-ethanol binge

Feeding mice with an HFD for 3 months followed by a binge of ethanol

KO

knockout

LD

lipid droplet

LPS

lipopolysaccharide

m

months

MPO

myeloperoxidase

NAFLD

nonalcoholic fatty liver disease

NASH

nonalcoholic steatohepatitis

PA

palmitic acid

PPAR γ peroxisome proliferation-activated receptor γ . TG, triglyceride**TG**

triglyceride

TNF

tumor necrosis factor

Tro

troglitazone

WT

wild-type

Figure 1. Hepatic *Pparg* deficiency reduces hepatic steatosis, but does not alleviate serum levels of ALT and AST following HFD-plus-binge ethanol

Pparg^{Hep-/-} mice and littermate WT controls were fed a HFD diet for 3 months, and then given one binge of ethanol. Blood and liver tissue samples were collected 9 hours post gavage for measurement. (A) Representative H&E staining of liver tissue sections are shown. (B) Serum and liver TG levels. (C) Serum ALT and AST levels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as indicated.

Figure 2. Hepatic *Pparg* deficiency increases *Cxcl1* expression and neutrophil infiltration following HFD-plus-ethanol binge

Pparg^{Hep-/-} and WT mice were set up for HFD-plus-binge ethanol model. Liver tissues were collected 9 hours post gavage for further analysis. (A) Representative MPO staining is shown (Red arrows indicate MPO⁺ cells). (B) Quantification of MPO⁺ cells/per 100× field was determined. (C, D) Total RNA was isolated and subjected to real-time PCR analysis of *Ly6g* (a marker of neutrophils) and other inflammatory genes. (E) Sera were collected to measure CXCL1 protein levels by using an ELISA kit. (F) Representative CXCL1 protein staining of liver tissue sections are shown (Black arrow indicates positive findings; Bar scale: 100 μm). **P*<0.05, ***P*<0.01, ****P*<0.001, as indicated.

Figure 3. Hepatic Fsp27, a *Pparg* downstream target gene, contributes to the pro-steatotic effect of PPAR γ in mice following HFD-plus-binge ethanol

(A) *Pparg*^{Hep-/-} and WT mice were set up for HFD-plus-binge ethanol model. Liver tissue samples were collected, and *Cidea*, *Cideb* and *Fsp27* mRNA expressions were determined by real-time PCR. (B-C) Male C57BL/6N mice were fed a HFD for 3 months, and administered with Ad-control-shRNA (10^9 Pfu) or Ad-sh*Fsp27* (10^9 Pfu) for the final 6 days. Ethanol (5g/kg) was given by oral gavage 9h before killing. Liver tissues and sera were collected for measurement. Liver histology was analyzed and serum ALT and AST levels were analyzed. (D-E) *Fsp27*^{Hep-/-} mice and WT mice were set up for HFD-plus-binge ethanol model. Liver histology is shown, and serum ALT and AST level were analyzed.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as indicated.

Figure 4. Hepatic *Fsp27* deletion does not affect neutrophil infiltration following HFD-plus-binge ethanol

(A, B) Male C57BL/6N mice were fed a HFD for 3 months, and administered with Ad-control-shRNA (10^9 Pfu) or Ad-sh*Fsp27* (10^9 Pfu) for the final 6 days. Ethanol (5g/kg) was given by oral gavage 9 hours before killing. Liver tissues and sera were collected for measurement. Representative MPO staining is shown in panel A. Quantification of MPO⁺ cells/per 100× field and real-time PCR analysis of *Ly6g* are shown in panel B. (C-D) *Fsp27^{Hep-/-}* mice and WT mice were set up for HFD-plus-binge ethanol model. Liver tissue samples were collected. Representative MPO staining is shown in panel C. Quantification of MPO⁺ cells/per 100× field and real-time PCR analysis of *Ly6g* mRNA are shown in panel D. (E) Real-time PCR analysis of *Cxcl1* mRNA in liver samples from panels A and C. Red arrows in panels A and C indicate MPO⁺ cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as indicated.

Figure 5. Hepatic *Pparg* deficiency attenuates liver fibrosis following HFD-plus-binge ethanol *Pparg*^{Hep-/-} and WT mice were set up for HFD-plus-binge ethanol model. Liver tissue samples were collected. (A) Representative Sirius red staining of liver tissue sections is shown. (B) Quantification of fibrotic area/per 100× field was determined. (C) Real-time PCR analyses of fibrosis-related genes. **P*<0.05, ***P*<0.01, ****P*<0.001, as indicated.

Figure 6. Interaction between PPAR γ and NF- κ B modulates FFAs-induced upregulation of *Cxcl1* in hepatocytes

(A, B) Primary hepatocytes from *Pparg*^{Hep-/-} mice and WT mice were isolated and stimulated with palmitic acid (PA, 0.3mM) or TNF- α (10ng/ml). Hepatocytes were collected and *Cxcl1* mRNA levels were determined by real-time qPCR. (C) Primary hepatocytes from C57BL/6N mice were isolated and stimulated with troglitazone (Tro, a PPAR γ agonist) and TNF- α (10ng/ml) for 12 hours, then cells were harvested and *Cxcl1* mRNA levels were determined by real-time PCR. (D) Primary hepatocytes from *Pparg*^{Hep-/-} mice and WT mice were isolated and stimulated by PA with pre-treatment by different doses of Bay-7085 (an NF- κ B inhibitor) for 30 minutes. *Cxcl1* mRNA expression was determined by real-time PCR. (E) HepG2 cells were stimulated by PA with PPAR γ agonist (Tro) or antagonist (GW9662) pretreatment. Cells were collected after the treatment at different time points, and *IL-8* mRNA levels were determined by real-time PCR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as indicated.

Figure 7. Acute ethanol binge suppresses hepatic *Pparg* activation and expression in HFD-fed mice

C57BL/6J male mice were divided into 4 groups, 2 groups were fed a chow diet and other 2 groups were fed a HFD diet for 3 months. On the last day of 3m-feeding, mice were given maltose or ethanol by oral gavage 9 hours before killing (Chow, Chow+binge, HFD, HFD +binge). Liver tissues were collected for further analysis. (A) Representative PPAR γ nuclear staining is shown (Red arrows indicate positive staining of hepatocyte nucleus). (B-C) Total hepatic protein and hepatic nuclear protein were extracted and subjected to Western blot analysis of total PPAR γ and nuclear PPAR γ protein. Representative blots are shown in panel B. Quantification of total PPAR γ / β -actin and nuclear PPAR γ /histone H3 is shown in panel C. (D) Real-time PCR analysis of *Pparg* mRNA was performed. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as indicated.

Figure 8. The dichotomy role of PPAR γ underlying the synergistic effect of HFD and acute ethanol binge on steatohepatitis

(A) Post HFD feeding, hepatic PPAR γ becomes activated and upregulates hepatic expression of FSP27, thereby promoting steatosis. PPAR γ also inhibits hepatic CXCL1 expression via the inhibition of NF- κ B, and subsequently reduces hepatic neutrophil infiltration. (B) Acute ethanol binge inhibits PPAR γ activation and subsequently diminishes the inhibitory effect of PPAR γ on the NF- κ B/CXCL1 axis, thus leading CXCL1 upregulation and massive hepatic neutrophil infiltration in HFD-fed mice. Acute ethanol also elevates FFA levels to further enhance the NF- κ B/CXCL1 pathway.