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Ligand activation of peroxisome proliferator-activated receptor- β/δ suppresses liver tumorigenesis in hepatitis B transgenic mice

Gayathri Balandaram^a, Lance R. Kramer^a, Boo-Hyon Kang^b, Iain A. Murray^a, Gary H. Perdew^a, Frank J. Gonzalez^c, and Jeffrey M. Peters^{a,*}

^aDepartment of Veterinary and Biomedical Sciences and The Center for Molecular Toxicology and Carcinogenesis, The Pennsylvania State University, University Park, Pennsylvania, USA

^bChemon Nonclinical Research Institute, 240 Nampyeong-ro, Yangji-myeon, Cheoin-gu, Yongin-si, Gyeonggi-do, Korea

^cLaboratory of Metabolism, National Cancer Institute, Bethesda, Maryland, USA

Abstract

Peroxisome proliferator-activated receptor- β/δ (PPAR β/δ) inhibits steatosis and inflammation, known risk factors for liver cancer. In this study, the effect of ligand activation of PPAR β/δ in modulating liver tumorigenesis in transgenic hepatitis B virus (HBV) mice was examined. Activation of PPAR β/δ in HBV mice reduced steatosis, the average number of liver foci, and tumor multiplicity. Reduced expression of hepatic CYCLIN D1 and c-MYC, tumor necrosis factor alpha (*Tnfa*) mRNA, serum levels of alanine aminotransaminase, and an increase in apoptotic signaling was also observed following ligand activation of PPAR β/δ in HBV mice compared to controls. Inhibition of *Tnfa* mRNA expression was not observed in wild-type hepatocytes. Ligand activation of PPAR β/δ inhibited lipopolysaccharide (LPS)-induced mRNA expression of *Tnfa* in wild-type, but not in *Ppar β/δ* -null Kupffer cells. Interestingly, LPS-induced expression of *Tnfa* mRNA was also inhibited in Kupffer cells from a transgenic mouse line that expressed a DNA binding mutant form of PPAR β/δ compared to controls. Combined, these results suggest that ligand activation of PPAR β/δ attenuates hepatic tumorigenesis in HBV transgenic mice by inhibiting steatosis and cell proliferation, enhancing hepatocyte apoptosis, and modulating anti-inflammatory activity in Kupffer cells.

* Corresponding Author: Jeffrey M. Peters, Ph.D. Department of Veterinary and Biomedical Sciences and The Center for Molecular Toxicology and Carcinogenesis, 312 Life Sciences Building. The Pennsylvania State University, University Park, PA 16802, USA. Tel: 814-863-1387; Fax: +1-814-863-1696; jmp21@psu.edu.

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Conflict of interest
None.

Keywords

Peroxisome proliferator-activated receptor- β/δ ; liver cancer; Kupffer cell; hepatitis B; inflammation

1. Introduction

PPAR β/δ is a member of the nuclear hormone receptor superfamily. Expression of PPAR β/δ is particularly high in skin, colon and liver and thought to be constitutively active in cells due to the presence of endogenous ligands (Bookout et al. 2006; Girroir et al. 2008; Modica et al. 2010). Consequently, PPAR β/δ dynamically modulates target gene expression through binding with peroxisome proliferator response elements (PPREs) of target genes. Nucleosome localization, chromatin structure, the presence/expression of co-repressors, co-activators, and enzymes that remodel chromatin all influence the binding of PPAR β/δ with DNA where it increases and decreases transcription of target genes (Khozoie et al. 2012). Additionally, PPAR β/δ can also modulate biological functions through protein-protein interactions with other transcription factors such as NF- κ B (Planavila et al. 2005a), STAT3 (Kino et al. 2007) and ERK5 (Woo et al. 2006).

Ligand activation of PPAR β/δ can prevent liver damage caused by a number of insults in multiple models. For example, steatosis and inflammation induced by feeding a methionine and choline deficient diet are attenuated in mice by ligand activation of PPAR β/δ with GW501516 (Nagasawa et al. 2006). This protective effect was mediated by increasing oxidation of fatty acids and inhibition of pro-inflammatory cytokines in the liver (Nagasawa et al. 2006), although it was also suggested that ligand activation of PPAR β/δ inhibits lipogenesis and prevents hepatic steatosis (Qin et al. 2008). Hepatotoxicity caused by exposure to azoxymethane or carbon tetrachloride is exacerbated in *Ppar β/δ* -null mice as compared to controls (Shan et al. 2008a), and this effect appears to be mediated by PPAR β/δ -dependent inhibition of NF- κ B-dependent induction of pro-inflammatory cytokines following administration of the PPAR β/δ ligand GW0742 (Shan et al. 2008b). Activation of PPAR β/δ with another PPAR β/δ ligand KD3010 can also prevent inflammation and fibrosis in mouse liver in response to exposure to carbon tetrachloride (Iwaisako et al. 2012). Additional data supporting the notion that PPAR β/δ protects the liver by promoting anti-inflammatory activities is provided by studies showing markedly enhanced expression of many target genes associated with inflammation in *Ppar β/δ* -null mice as compared to controls (Sanderson et al. 2010). Since steatosis, inflammation and fibrosis are compounding risk factors for hepatocarcinogenesis, it is of interest to note that the role of PPAR β/δ in liver cancer has not been examined to date using an in vivo model.

Hepatocarcinogenesis is one of the leading causes of death in human cancer patients (Ferlay et al. 2015). Approximately 600,000 humans die every year due to liver cancer that develops from hepatitis B virus (HBV) infection (Franco et al. 2012). Chronic HBV infection induces persistent hepatocyte inflammation, oxidative DNA damage, uncontrolled cellular proliferation, fibrosis and cirrhosis, which eventually culminates in hepatocellular carcinoma (Park et al. 2007). Since steatosis and inflammation are two important mechanisms that

mediate HBV-dependent hepatocellular carcinoma, and ligand activation of PPAR β/δ prevents hepatic steatosis and inflammation, the present study examined the hypothesis that ligand activation of PPAR β/δ inhibits liver cancer in HBV transgenic mice.

2. Material and methods

2.1. Mouse lines

Male C57BL/6J transgenic mice (Tg(Alb1-HBV)Bri44) that express the human HBV large envelope protein driven by the mouse albumin promoter (Chisari et al. 1987) and male C57BL/6J control mice were obtained from The Jackson Laboratory (Bar Harbor, ME). These HBV transgenic mice over-express the human HBV large envelope protein in hepatocytes, which leads to inflammation, hyperplasia and liver cancer (Chisari et al. 1989). Male wild-type and *Ppar β/δ* -null mice used for these studies have been previously described (Peters et al. 2000). A transgenic mouse line expressing a previously described PPAR β/δ isoform containing a DNA binding domain mutation (DBM) (Shi et al. 2002) was generated. The PPAR β/δ DBM isoform was produced by site directed mutagenesis of a mouse PPAR β/δ expression vector (pSG5-PPAR β/δ ; kindly provided by Drs. Pallavi Devchand and Walter Wahli). The 90th and 93rd codons of this cDNA encoding critical cysteine residues in the DNA binding domain of PPAR β/δ were mutated from TGC to GCC, such that the resulting PPAR β/δ DBM expressed alanine residues rather than cysteine residues in the expressed protein. The mutant PPAR β/δ DBM expression vector was then characterized to verify that the PPAR β/δ DBM was incapable of binding with its cognate DNA response element, an event required for direct regulation of PPAR β/δ target genes. This characterization included: 1) examining the ability of the PPAR β/δ DBM to activate a luciferase reporter assay in wild-type and *Ppar β/δ* -null primary keratinocytes as previously described (Kim et al. 2006) except that a luciferase construct containing four copies of the rat acyl-CoA oxidase peroxisome proliferator response element (PPRE) was used for the reporter, 2) verifying that expression levels of the transfected wild-type PPAR β/δ and PPAR β/δ DBM were equivalent in transfected keratinocytes using quantitative western blot analysis as previously described (Girroir et al. 2008), 3) electrophoretic mobility shift analysis (EMSA) to determine whether the PPAR β/δ DBM can or cannot bind to a PPRE in the presence of its heterodimerization partner, retinoid X receptor- α (RXR α) as previously described (Kim et al. 2004), 4) determining whether in vitro translation efficiency of both wild-type PPAR β/δ and the PPAR β/δ DBM used for the EMSA was similar using a previously described method (Girroir et al. 2008), and 5) determining whether both the wild-type PPAR β/δ and the PPAR β/δ DBM can heterodimerize with RXR α using co-immunoprecipitation analysis as previously described (Sumanasekera et al. 2003). After this characterization, the cDNA encoding the mouse PPAR β/δ DBM cDNA was subcloned in frame into a vector with the CD68 promoter (kindly provided by Dr. Pamela Hankey-Giblin). This transgene vector was linearized, and microinjected into pronuclear stage embryos from homozygous *Ppar β/δ* -mice at the Penn State Transgenic Mouse Facility (University Park, PA). After transfer to pseudo-pregnant CD-1 female mice, transgenic offspring were screened using Southern blot analysis of tail DNA isolated from the progeny using a previously described method (Peters et al. 2000) and a cDNA probe encoding part of the CD68 promoter and the PPAR β/δ DBM cDNA. The CD68 promoter is highly active in Kupffer cells. Confirmed transgenic offspring

that incorporated the PPAR β / δ DBM cDNA into their genome were bred using a brother/sister mating strategy to obtain homozygous transgenic mice. Functional characterization of the PPAR β / δ DBM transgenic mice was performed as described below for examination of primary Kupffer cells.

2.2. Mouse treatments

All animal experiments were approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University. To determine the effect of ligand activation of PPAR β / δ on tumorigenicity in HBV transgenic mice, 7-month-old male HBV transgenic mice were administered the specific PPAR β / δ agonist GW0742 at a dose of 2.5 mg/kg (5 days per week) for 8 months. Mice were dosed with a pellet composed of bacon-flavored transgenic dough (Bioserv, Inc., Piscataway, NJ) or the bacon-flavored transgenic dough mixed with GW0742 at a dose of 2.5 mg/kg (N = 5 mice per group). After 8 months of treatment, mice were euthanized by overexposure to carbon dioxide and exsanguination followed by cervical dislocation. The total number of visible tumors was counted for each mouse liver. Sections of liver tissue with and without visible tumors were fixed in 10% phosphate buffered formalin (PBF). Sections of liver without visible tumors were also snap frozen, stored at -80°C until further use. Serum was isolated from whole blood. To determine the sub-chronic effect of ligand activation of PPAR β / δ in HBV transgenic mice, 6-month-old male mice were administered GW0742 at a dose of 5.0 mg/kg (5 days per week) for 3 weeks.

2.3. Histopathological analyses

Sections of liver tissue were fixed in 10% PBF overnight and transferred to 70% ethanol, prior to embedding in paraffin for histological analysis. Tissue sections were stained with hematoxylin and eosin. The total number of steatotic regions was quantified in the left lobe of representative liver sections using light microscopy. A pathologist scored the total number of liver tumor foci from representative liver sections.

2.4. RNA isolation and quantitative polymerase chain reaction (qPCR) analysis

RNA was extracted from whole liver tissue or cultured cells using RiboZol RNA extraction reagent (Amresco Inc., Solon, OH) and 1.25 μg of total RNA was reverse transcribed into cDNA using reverse transcription kit (Promega, WI.). qPCR reactions were performed using MyiQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) to quantify mRNA encoding the PPAR β / δ target genes adipocyte differentiation-related protein (*Adrp*), angiopoietin-like 4 (*Angptl4*), and tumor necrosis factor alpha (*Tnfa*). Relative mRNA expression was normalized to internal control values of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) mRNA. The following oligonucleotide primers were used for: (*Adrp*) forward, 5'-CACAAATTGCGGTTGCCAAT-3' and reverse, 5'-ACTGGCAACAATCTCGGACGT-3'; (*Angptl4*) forward, 5'-TTCTCGCCTACCAGAGAAGTTGGG-3' and reverse 5'-CATCCACAGCACCTACAACAGCAC-3'; (*Tnfa*) forward, 5'-AGGGTCTGGGCCATAGAACT-3' and reverse, 5'-CCACCACGCTCTTCTGTCTAC-3'; and (*Gapdh*) forward, 5'-TCAACAGCAACTCCCACTCTTCCA-3' and reverse 5'-ACCCTGTTGCTGTAGCCGTATTCA-3'.

2.5. Protein isolation and quantitative western blot analysis

Liver tissues were homogenized, resolved using SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane using electroblotting as previously described (Yao et al. 2014). Membranes were blocked with 5% non-fat milk or 0.5% gelatin in Tris buffered saline with Tween-20 (TBST) and subsequently incubated with primary and secondary antibodies. After incubation with primary or secondary antibodies, membranes were washed three times with TBST. The primary antibodies used were: anti-CYCLIN B1 (SC-752), anti-CYCLIN D1 (SC-753), anti-cMYC (SC-764) and anti- β -ACTIN (SC-47778) from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA); Anti-PARP (CST #9542S) from Cell Signaling Technology (Danvers, MA) and anti-LDH (Rockland, Gilbertsville, PA). Biotinylated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). After incubation with the secondary antibody and washing with TBST, the membranes were incubated with 125 I-labeled streptavidin, prepared as previously described (Singh et al. 1996), and then washed three times with TBST. Membranes were then exposed to phosphorimager plates and the level of radioactivity quantified with filmless autoradiographic analysis using a Packard Phosphorimager (PerkinElmer, Waltham, MA). The relative expression level of specific proteins was normalized to that of LDH or ACTIN and are presented as fold change compared to controls.

2.6. Serum alanine aminotransferase (ALT) analysis

Serum levels of ALT were determined by spectrophotometric analysis utilizing the RAICHEM ALT assay kit (Cliniqa Corporation, San Marcos, CA) according to manufacturer's protocol.

2.7. Isolation of primary Kupffer cells and hepatocytes

Kupffer cells and hepatocytes from mice 12–16 week-old mice were isolated using a previously described method (Smedsrod et al. 1985) with minor modifications. Mice were anesthetized by intraperitoneal injection of avertin prior to liver perfusion. Briefly, whole livers were perfused with buffer I (142 mM sodium chloride, 6.4 mM potassium chloride, 9.6 mM HEPES, 30 mM sodium hydroxide), followed by buffer II (buffer I supplemented with 4.76 mM calcium chloride and collagenase type IV (Worthington Biochemical Corporation, Lakewood, NJ)) via the inferior vena cava, prior to excision. Perfused livers were then minced and suspended in ice-cold buffer III (buffer I with 1% bovine serum albumin) and then filtered through a 70 μ m nylon cell strainer. The filtered cells were further purified for cell culture via centrifugation twice at $54 \times g$ for 3 minutes at 4 °C to isolate the hepatocyte pellet. The supernatants were further centrifuged at $900 \times g$ for 10 minutes at 4 °C. The resulting cell pellet was then re-suspended in buffer III and centrifuged through a discontinuous gradient of 25% and 50% Percoll (Sigma-Aldrich, St. Louis, MO) at 4°C. The Percoll fraction containing the purified non-parenchymal cells was then washed once with buffer III and the Kupffer cells were enriched by selective adherence to tissue culture plates.

Isolated Kupffer cells and hepatocytes were cultured in RPMI medium containing L-glutamine (Mediatech Inc., Manassas, VA), 10% fetal bovine calf serum and 1% penicillin and streptomycin at 37°C with 5% carbon dioxide. The purity of isolated Kupffer cells was

determined by their ability to phagocytize latex beads. Isolated primary Kupffer cells and hepatocytes were cultured for 48 hours. After this 48 hours, the media was changed to control media or media containing 1 μ M GW0742 and the cell cultured for an additional 23 hours. After this 23 hours, the media was changed to control media or media containing 1 μ M GW0742 with or without 10 ng/mL lipopolysaccharide (LPS from *Escherichia coli*, 0111:B4, Sigma-Aldrich, St. Louis, MO) for 1 hour.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 5 (GraphPad Software, Inc., La Jolla, CA). For comparison of more than two groups, analysis of variance using Tukey's multiple comparison tests was used. Student's unpaired t-test was used for comparing differences between two groups. The data are shown as mean $\pm$ S.E.M. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Ligand activation of PPAR β/δ attenuates hepatic tumorigenesis in HBV mice

Ligand activation of PPAR β/δ in the liver of HBV mice treated with GW0742 for 7 months was confirmed by an increase in expression of the PPAR β/δ target gene *Angptl4* as compared to control (Fig. 1A). Histopathological analyses of the liver of 15 month-old mice revealed that control HBV mice had $\sim$ 20 foci/section and that ligand activation of PPAR β/δ in HBV mice caused a significant decrease in the average number of foci/liver section as compared to controls (Fig. 1B). Foci were not observed in control C57Bl/6J mouse liver (Fig. 1B). Control HBV transgenic mice also exhibited severe hepatomegaly at 15 months of age and this effect was not observed in 15 month-old HBV transgenic mice following ligand activation of PPAR β/δ for 7 months (data not shown). Grossly apparent tumor nodules were present in control 15 month-old HBV mice and this effect was markedly attenuated in HBV mice treated with the PPAR β/δ ligand GW0742. No grossly visible tumors were noted in control C57Bl/6J mice. Histopathological analysis was consistent with gross observations, as tumor multiplicity was reduced by ligand activation of PPAR β/δ in liver of HBV mice compared to control C57BL/6J and HBV mice (Fig. 1C). Histopathological analysis of liver tissue also revealed marked hepatic lipid accumulation in HBV mice as compared to liver of HBV mice following ligand activation of PPAR β/δ (Fig. 2A, B).

3.2. Ligand activation of PPAR β/δ inhibits hepatic markers of cell proliferation and enhances apoptotic signaling in HBV mice

Quantitative western blot analysis of liver was performed to examine the effect of ligand activation of PPAR β/δ on markers of cell proliferation and apoptosis. There was a trend of reduced expression of CYCLIN B1 expression by ligand activation of PPAR β/δ in HBV mouse liver as compared to control, but this effect was not statistically significant (Fig. 3A). By contrast, ligand activation of PPAR β/δ caused a significant reduction in expression of CYCLIN D1 and c-MYC in HBV mouse liver and this effect was not found in control HBV mouse liver (Fig. 3A). To determine the effect of ligand activation of PPAR β/δ on apoptotic signaling in this model, relative PARP cleavage was assessed. An increase in the ratio of

cleaved to uncleaved PARP was observed in HBV mouse liver following ligand activation of PPAR β/δ , and this increase was not observed in control HBV mouse liver (Fig. 3B).

3.3. Ligand activation of PPAR β/δ protects against hepatotoxicity and inhibits expression of hepatic *Tnfa* in HBV mice

Previous studies showed that ligand activation of PPAR β/δ mitigates liver toxicity induced by carbon tetrachloride by inhibiting expression of pro-inflammatory cytokines (Shan et al. 2008a; Shan et al. 2008b). To determine if a similar PPAR β/δ -dependent mechanism contributed to the decrease in liver tumorigenicity observed in HBV mice following ligand activation of PPAR β/δ , serum markers of liver damage and expression of *Tnfa* mRNA were examined in HBV mice before liver tumors develop (Chisari et al. 1989). Indeed, ligand activation of PPAR β/δ caused a significant reduction of serum ALT, a marker of liver injury, and expression of *Tnfa* mRNA, a marker of hepatic inflammation, in HBV mice as compared to controls (Fig. 4A, B).

3.4. Decreased hepatic *Tnfa* mRNA by ligand activation of PPAR β/δ is not modulated in hepatocytes

TNF α is a pro-inflammatory cytokine that can promote liver cancer by enhancing cell proliferation (Park et al. 2010; Wheelhouse et al. 2003). Thus, inhibition of TNF α expression could contribute to the mechanisms by which ligand activation of PPAR β/δ attenuates liver tumorigenesis in HBV transgenic mice. Ligand activation of PPAR β/δ increased expression of the PPAR β/δ target gene *Adrp* in primary hepatocytes from wild-type mice, demonstrating that hepatocytes express a functional PPAR β/δ (Fig. 5A). However, ligand activation of PPAR β/δ did not modulate LPS-induced expression of *Tnfa* mRNA in primary hepatocytes from wild-type mice (Fig. 5B). This is consistent with previous studies demonstrating that the anti-inflammatory activity of PPAR β/δ is not directly mediated by PPAR β/δ in hepatocytes.

3.5. PPAR β/δ -dependent suppression of hepatic *Tnfa* is independent of PPAR β/δ interacting with its cognate DNA binding domain

Kupffer cells are pro-inflammatory cells of the liver and known to increase expression of TNF α in response to hepatocyte damage (Jang et al. 2014). Previous studies have established that one mechanism by which PPAR β/δ can inhibit expression of NF- κ B target genes such as *Tnfa* is by physically interacting with the p65 subunit of the NF- κ B complex, thereby attenuating expression of *Tnfa* (Kilgore and Billin 2008). To examine this mechanism in an in vivo model, a transgenic mouse expressing a PPAR β/δ DBM in Kupffer cells was created. Transfection experiments using mouse wild-type and *Ppar β/δ* -null primary keratinocytes showed that the PPAR β/δ DBM is incapable of activating a PPRE-driven reporter construct (Fig. 6A). Quantitative western blot analysis demonstrated that expression of both wild-type and the PPAR β/δ DBM in the transfected keratinocytes (Fig. 6A) was equivalent (Fig. 6B). EMSA analysis was performed and showed that while in vitro translated PPAR β/δ can bind to a PPRE in the presence of its heterodimerization partner, RXR α (lane 1 versus lane 3), binding of the PPAR β/δ DBM to the PPRE does not occur (lane 3 versus lane 9; Fig. 6C). Quantitative western blot analysis demonstrated that expression of the in vitro translated wild-type PPAR β/δ and the PPAR β/δ DBM used for the

EMSA was similar (Fig. 6C). Lastly, immunoprecipitation analysis demonstrated that both the wild-type PPAR β/δ and the PPAR β/δ DBM can heterodimerize with RXR α (Fig. 6D). A transgenic mouse line that expressed the PPAR β/δ DBM was generated as described in Materials and Methods and Kupffer cells were isolated from these mice, as well as wild-type and *Ppar β/δ* -null mice. Ligand activation of PPAR β/δ increased expression of the PPAR β/δ target gene *Adrp* in primary Kupffer cells isolated from wild-type mice, but not in *Ppar β/δ* -null Kupffer cells or Kupffer cells isolated from the transgenic mouse line expressing the PPAR β/δ DBM (Fig. 6E). LPS-induced expression of *Tnfa* mRNA in primary Kupffer cells isolated from wild-type mice, *Ppar β/δ* -null and the transgenic mice expressing the PPAR β/δ DBM, but this effect was markedly enhanced in the *Ppar β/δ* -null Kupffer cells (Fig. 6E). Moreover, ligand activation of PPAR β/δ attenuated expression of *Tnfa* mRNA in Kupffer cells from wild-type and the transgenic mice expressing the PPAR β/δ DBM, but this effect was not observed in Kupffer cells treated with LPS and GW0742 from *Ppar β/δ* -null mice (Fig. 6E).

4. Discussion

Results from the present study are the first to demonstrate that ligand activation of PPAR β/δ attenuates liver tumorigenesis in HBV transgenic mice. Results from published studies examining other cancer models also show that ligand activation of PPAR β/δ inhibits pre-neoplastic stages including steatosis, cell proliferation and inflammation, which contribute to the progression of hepatocellular carcinoma (reviewed in (Peters et al. 2015; Peters et al. 2011; Peters et al. 2012)). This is consistent with results from the present study that also demonstrate that ligand activation of PPAR β/δ inhibits steatosis, together with gene expression linked with cell proliferation, inflammation and promotion of apoptosis in the liver of HBV mice.

The decrease in serum ALT and hepatic *Tnfa* mRNA by ligand activation of PPAR β/δ in HBV transgenic mice is consistent with the PPAR β/δ -dependent hepatoprotective effects observed previously in mice treated with carbon tetrachloride (Shan et al. 2008b). Results from the present study also demonstrate another antiinflammatory role for PPAR β/δ in response to a different type of hepatic injury. In an effort to determine the specific cell type involved, the present study shows that PPAR β/δ does not modulate anti-inflammatory activities in the hepatocytes, which is also consistent with previous findings (Shan et al. 2008b). Notably, a PPAR β/δ -dependent inhibition of *Tnfa* mRNA was observed in Kupffer cells, and this effect did not require binding of PPAR β/δ to its cognate DNA binding domain as shown by examination of transgenic mice expressing a PPAR β/δ DBM. These results support numerous studies showing that PPAR β/δ suppresses inflammation in the liver (Lee et al. 2012; Lim et al. 2009; Sanderson et al. 2010; Shan et al. 2008a; Shan et al. 2008b) and in other models (Barish et al. 2008; Collino et al. 2011; Defaux et al. 2009; Haskova et al. 2008; Salvado et al. 2014). However, they markedly extend previous studies by suggesting that PPAR β/δ expression in Kupffer cells is required to modulate the anti-inflammatory and chemopreventive activity observed in HBV transgenic mice, and that this is mediated through a mechanism that does not require binding to its cognate DNA binding element found in its target genes. The anti-inflammatory activities of PPAR β/δ could be mediated by suppression of NF- κ B activity as a result of direct binding of PPAR β/δ to the p65 subunit of

NF- κ B (Alvarez-Guardia et al. 2011; Ding et al. 2006; Planavila et al. 2005b; Zingarelli et al. 2010).

Hepatic steatosis is commonly observed with viral hepatitis infection (Mihm et al. 1997; Peng et al. 2008) and is also a risk factor for liver cancer (Ohata et al. 2003; Takuma et al. 2007). The observed decrease in hepatic steatosis in HBV mice following ligand activation of PPAR β/δ is consistent with other studies (Lim et al. 2009; Staels et al. 2013). One mechanism not examined in the present work is that PPAR β/δ up-regulates a target gene *Insig-1* that in turn inhibits activation of the lipogenic transcription factor SREBP-1, thereby reducing hepatic steatosis (Qin et al. 2008). Interestingly, there is also a strong link between TNF α expression and the development of hepatic steatosis. For example, studies have shown that TNF α induces hepatic lipid accumulation and that inhibition of TNF α signaling reduces hepatic steatosis in steatohepatitis and diet-induced obesity models (Barbuio et al. 2007; Endo et al. 2007; Koca et al. 2008). In particular, TNF α secreted by activated Kupffer cells may facilitate hepatic steatosis as deletion of Kupffer cell specific TNF α or inhibition of TNF α activity in Kupffer cells decreases hepatic lipid accumulation (Huang et al. 2010; Tosello-Tramont et al. 2012). Thus, the PPAR β/δ -dependent inhibition of TNF α could contribute to the reduction hepatic steatosis observed in HBV mice in response to ligand activation of PPAR β/δ . Consistent with this, a recent study showed that PPAR β/δ inhibits hepatic steatosis and is associated with inhibition of TNF α expression in liver (Lee et al. 2012).

One possible mechanism by which ligand activation of PPAR β/δ inhibits cell proliferation in HBV mice is through inhibition of expression of CYCLIN D1 and c-MYC, however the precise mechanism was not determined in this study. Previous work has shown that ligand activation of PPAR β/δ can promote terminal differentiation, which is known to be associated with a concomitant withdrawal from the cell cycle (reviewed in (Peters et al. 2015; Peters et al. 2011; Peters et al. 2012)). Other studies have demonstrated that ligand activation of PPAR β/δ can inhibit cell proliferation by causing a block in mitosis (Zhu et al. 2012). TNF α is a mitogen in the liver (Diaz-Gil et al. 2003) and has been strongly linked to the development of hepatocellular carcinoma (Knight et al. 2000; Park et al. 2010). TNF α secreted from activated Kupffer cells can cause activation of NF- κ B in hepatocytes and promote liver cancer in a cholestatic hepatitis model, and treatment with TNF α neutralizing antibodies prevents tumor progression in this model (Pikarsky et al. 2004). TNF α is an activator of NF- κ B and both CYCLIN D1 and c-MYC that promote cell proliferation and are known target genes of NF- κ B (Guttridge et al. 1999; La Rosa et al. 1994). CYCLIN D1 regulates the G1/S phase transition during cell cycle (Resnitzky et al. 1994) and can promote hepatocyte proliferation in the absence of a mitogen (Albrecht and Hansen 1999). CYCLIN D1 is also frequently over-expressed in liver tumor models and can contribute to the progression of hepatitis-induced hepatocellular carcinoma (Chen et al. 2015; Masaki et al. 2003; Nishida et al. 1994). c-MYC, which is often over-expressed in liver cancer (Jang et al. 2012; Kawate et al. 1999), can also inhibit termination of differentiation, and promote hepatocyte proliferation, angiogenesis and cellular transformation, all of which can contribute to tumor promotion (Meyer and Penn 2008). Inhibition of both CYCLIN D1 and c-MYC expression also inhibits proliferation of both human and rodent cancer cell lines (Simile et al. 2004). Further, knockout of c-MYC inhibits hepatocellular proliferation caused

by peroxisome proliferators and diethylnitrosamine (Qu et al. 2014). Thus, it remains possible that inhibition of CYCLIN D1 and c-MYC by ligand activation of PPAR β/δ might have in part contributed to the observed inhibition in tumor progression by reducing the proliferative potential of hepatocytes in HBV transgenic mice. Further studies are needed to determine how PPAR β/δ modulates cellular function to cause a decrease in the expression of proteins that promote cell proliferation in the liver.

In contrast to the observations in the HBV mouse model in vivo, it was also reported that ligand activation of PPAR β/δ promotes proliferation (Glinghammar et al. 2003; Xu et al. 2006a) inhibits proliferation (Vacca et al. 2014), or has no effect on proliferation of human liver cancer cell lines (Hollingshead et al. 2007). That ligand activation of PPAR β/δ promotes proliferation of human cholangiocarcinoma cell lines has also been reported (Han et al. 2008; Xu et al. 2006b). Two mutually exclusive mechanisms were proposed to explain this increase in cell proliferation: 1) that ligand activation of PPAR β/δ increases cyclooxygenase-2 (COX2) activity that in turn drives cell growth of human liver cancer cell lines (Glinghammar et al. 2003; Xu et al. 2006a; Xu et al. 2006b), or 2) that enhanced WNT/ β -CATENIN signaling drives cells growth of human cholangiocarcinoma cell lines (Han et al. 2008). However, it is critical to note that both of these hypothetical mechanisms are reported in peer-reviewed manuscripts that have since been retracted due to misuse of data (Han et al. 2015; Xu et al. 2015). Thus, observations made in human liver cancer cell lines suggesting that ligand activation of PPAR β/δ promotes proliferation that are predominantly not scientifically valid (Xu et al. 2006a; Xu et al. 2006b) cannot be compared with the results from the present studies. However, it is worth noting that the present studies are consistent with an in vitro study indicating that ligand activation of PPAR β/δ inhibits proliferation of a human liver cancer cell line (Vacca et al. 2014).

In summary, results from the present study strongly support earlier work on liver cell proliferation and carcinogenesis in the HBV model and are in agreement with earlier studies demonstrating anti-inflammatory, anti-proliferative and anti-steatotic mechanisms mediated by PPAR β/δ in the liver. Additionally, the findings presented here have revealed that PPAR β/δ -dependent signaling in Kupffer cells likely suppresses hepatic tumorigenesis in an in vivo model that requires inflammation and steatosis to induce liver cancer.

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Abbreviations

ALT	alanine aminotransaminase
CCl ₄	carbon tetrachloride
COX-2	cyclooxygenase-2

DBM	DNA binding domain mutant
ERK5	extracellular signal-regulated kinase 5
hepatitis B virus	HBV
LPS	lipopolysaccharide
NF-κB	nuclear factor κ B
PARP	poly (ADP-ribose) polymerase
PBF	phosphate buffered formalin
PPARβ/δ	peroxisome proliferator-activated receptor- β/δ
PGE₂	prostaglandin E ₂
STAT3	signal transducer and activator of transcription 3
TNFα	tumor necrosis factor- α

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Highlights

- The role of PPAR β/δ in HBV-induced liver cancer was examined.
- PPAR β/δ inhibits steatosis, inflammation, tumor multiplicity and promotes apoptosis.
- Kupffer cell PPAR β/δ mediates these effects independent of DNA binding.

Fig. 1. Ligand activation of PPAR β/δ suppresses liver tumorigenesis in HBV transgenic mice HBV transgenic mice were treated with or without the PPAR β/δ agonist GW0742 for 8 months (2.5 mg/kg, 5 days per week). (A) Increased hepatic expression of angiopoietin-like 4 protein (*Angptl4*) mRNA demonstrates effective activation of PPAR β/δ by GW0742 in HBV transgenic mice. (B) Ligand activation of PPAR β/δ suppresses the average number of hepatic foci in HBV transgenic mice as compared to C57BL/6J and HBV transgenic control mice. (C) Ligand activation of PPAR β/δ inhibits tumor multiplicity in HBV transgenic mice as compared to C57BL/6J and HBV transgenic control mice. Values represent the mean $\pm$

S.E.M. N = 5 mice per group. *Significantly different than HBV control, $P < 0.05$. Values with different letters are significantly different, $P < 0.05$.

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Fig. 2. Inhibition of hepatic steatosis by ligand activation of PPAR β/δ in HBV transgenic mice HBV transgenic mice were treated with or without the PPAR β/δ agonist GW0742 for 8 months (2.5 mg/kg, 5 days per week). (A) Representative photomicrographs of hematoxylin and eosin stained liver sections from three HBV transgenic control mice or HBV transgenic mice treated with the PPAR β/δ ligand GW0742 for 8 months (2.5 mg/kg, 5 days per week). (B) Quantification of hepatic steatotic regions in HBV transgenic control mice and HBV transgenic mice treated with the PPAR β/δ ligand GW0742 as described above. Values

represent the mean $\pm$ SEM. N = 5 mice per group. *Significantly different than HBV control, $P = 0.05$.

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Fig. 3. Ligand activation of PPAR β/δ inhibits expression of markers of cell proliferation and increases apoptosis in the liver of HBV transgenic mice

(A) Quantitative western blot analyses of CYCLIN B1, CYCLIN D1 and c-MYC, and (B) uncleaved (U) and cleaved PARP (C) in the liver of HBV transgenic mice treated with or without the PPAR β/δ agonist GW0742 for 8 months (2.5 mg/kg, 5 days per week). Relative expression of CYCLIN B1, CYCLIN D1 and c-MYC were normalized to expression of LDH and are presented as the mean $\pm$ S.E.M.. The ratio of the normalized expression of cleaved PARP to uncleaved PARP (C/U) is shown as a marker of relative apoptosis. Values

represent the mean $\pm$ S.E.M. N = 3 representative, independent mouse livers per group.

*Significantly different than HBV control, $P = 0.05$.

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Fig. 4. Ligand activation of PPAR β/δ inhibits liver toxicity and hepatic expression of the pro-inflammatory cytokine *Tnfa* mRNA in HBV transgenic mice

(A) Average serum ALT in control HBV transgenic mice and HBV transgenic mice treated with the PPAR β/δ agonist GW0742 for 3 weeks (5 mg/kg, 5 days per week). (B) Hepatic expression of *Tnfa* mRNA in control HBV transgenic mice and HBV transgenic mice treated with the PPAR β/δ agonist GW0742 as described above. Values represent the mean $\pm$ S.E.M.. N = 3 representative, independent samples per group. *Significantly different than HBV control, $P = 0.05$.

Fig. 5. Ligand activation of PPAR β/δ does not influence LPS-induced pro-inflammatory cytokine *Tnfa* mRNA expression in hepatocytes

Primary hepatocytes were isolated from wild-type mice as described. (A) Ligand activation of PPAR β/δ increases expression of *Adrp* mRNA in primary hepatocytes. (B) LPS induces expression of *Tnfa* mRNA while ligand activation of PPAR β/δ does not inhibit LPS-induced expression of *Tnfa* mRNA in primary hepatocytes. Values represent the mean $\pm$ S.E.M. with a minimum of N = 3 independent replicates per treatment group. *Significantly different than control, $P < 0.05$.

Fig. 6. PPARβ/δ-dependent repression of LPS-induced expression of the proinflammatory cytokine *Tnfa* mRNA by ligand activation of PPARβ/δ in Kupffer cells does not require binding to its cognate response element

Characterization of a DNA binding domain mutant (DBM) isoform of PPARβ/δ in vitro. Mouse primary keratinocytes from wild-type (+/+) or *Pparβ/δ*-null (-/-) transfected with a control vector, a wild-type PPARβ/δ expression vector, or a PPARβ/δ DBM expression vector, with or without the PPARβ/δ ligand GW0742. (A) The PPARβ/δ DBM is incapable of activating a PPRE-driven reporter construct. (B) Expression of PPARβ/δ in the controls and PPARβ/δ DBM in the transfected keratinocytes in panel (A). Relative expression was

normalized to LDH. (C) EMSA analysis demonstrates that PPAR β/δ binds to a PPRE in the presence of its heterodimerization partner, RXR α . By contrast, binding of the PPAR β/δ DBM to the PPRE does not occur. The right panel of (C) demonstrates that the in vitro translation of both wild-type PPAR β/δ and the PPAR β/δ DBM used for the EMSA is similar. (D) Immunoprecipitation analysis demonstrates that both the wild-type PPAR β/δ and the PPAR β/δ DBM can heterodimerize with RXR α . (E) Expression of the target gene *Adip* mRNA is increased in Kupffer cells isolated from (+/+) mice, but not in Kupffer cells isolated from (-/-) mice or a transgenic mouse line expressing the PPAR β/δ DBM (DBM) by ligand activation with the PPAR β/δ ligand GW0742. (F) LPS-induced expression of *Tnfa* mRNA is repressed by ligand activation of PPAR β/δ in Kupffer cells isolated from (+/+) mice or a transgenic mouse line expressing the PPAR β/δ DBM, but not in Kupffer cells isolated from (-/-) mice. Values represent the mean $\pm$ S.E.M. with a minimum of N = 3 independent replicates per treatment group. *Significantly different than control, $P < 0.05$.