Research Article

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Cell-specific PPARγ deficiency establishes anti-inflammatory and anti-fibrogenic properties for this nuclear receptor in non-parenchymal liver cells

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Background & Aims: PPARγ plays an essential role in the transcriptional regulation of genes involved in lipid and glucose metabolism, insulin sensitivity, and inflammation. We recently demonstrated that PPARγ plays a causative role in hepatocyte lipid deposition, contributing to the pathogenesis of hepatic steatosis. In this study, we investigated the role of PPARγ in the inflammatory and fibrogenic response of the liver.

Methods: Heterozygous floxed/null Cre/LoxP mice with targeted deletion of *PPARγ* in either hepatocytes (*Alb*-Cre), macrophages (*LysM*-Cre) or hepatic stellate cells (HSCs) (*aP2*-Cre) were submitted to carbon tetrachloride (CCl_4) liver injury. Further analyses were performed in precision-cut liver slices (PCLS) and primary cultures of hepatocytes, macrophages, and HSCs.

Results: *LysM*-Cre mice displayed an exacerbated response to chronic CCl_4 injury and showed higher necroinflammatory injury, lipid peroxidation, inflammatory infiltrate, cleaved-caspase-3 and caspase 3/7 activity, and COX-2, TNF-α, CXCL2, and IL-1β expression than *Alb*-Cre and control mice. The deleterious effects of PPARγ disruption in liver macrophages were confirmed in an acute model of CCl_4 injury as well as in PCLS incubated with LPS. Moreover, *LysM*-Cre mice showed an aggravated fibrogenic response to CCl_4, as revealed by more prominent Sirius Red and Masson's trichrome staining, elevated hydroxyproline content and induced α-SMA and TIMP-1 expression. Importantly, *aP2*-Cre mice with specific disruption of PPARγ in HSCs, as confirmed by immunocytochemical analysis of individual liver cells, also showed exacerbated liver damage and fibrogenic response to CCl_4.

Conclusions: These data unveil anti-inflammatory and anti-fibrogenic roles for PPARγ in non-parenchymal liver cells.

Introduction

There is strong evidence that peroxisome proliferator-activated receptor (PPAR)γ is a fundamental transcription factor controlling anabolic functions such as adipogenesis, fat deposition, and insulin sensitivity [1]. A large amount of literature also implicates PPARγ in the regulation of the inflammatory response [2]. Indeed, PPARγ ligands such as thiazolidinediones (TZD) have been shown to inhibit the production of inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in monocytes, macrophages, and epithelial cells [2]. Consistent with these properties, administration of PPARγ ligands to experimental models has been shown to ameliorate inflammation in the pancreas, lungs, joints, nervous system, and gastrointestinal tract [1].

The role of PPARγ in the regulation of hepatic inflammatory and fibrogenic response is still an open question. While *in vitro* findings postulate PPARγ as anti-inflammatory and anti-fibrogenic, *in vivo* studies do not clearly establish this. Indeed, initial discoveries in hepatic stellate cells (HSCs) demonstrated that

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Abbreviations: PPARγ, peroxisome proliferator-activated receptor γ; TZD, thiazolidinediones; TNF-α, tumor necrosis factor α; IL, interleukin; HSCs, hepatic stellate cells; NAFLD, non-alcoholic fatty liver disease; CCl_4, carbon tetrachloride; LPS, lipopolysaccharide; PCLS, precision-cut liver slices; *Alb*-Cre, albumin-Cre (hepatocyte-specific PPARγ deficient mice); *LysM*-Cre, lysozyme M-Cre (macrophage-specific PPARγ deficient mice); *aP2*-Cre, adipocyte fatty acid-binding protein 4-Cre (HSC-specific PPARγ deficient mice); LDH, lactate dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; NF-κB, nuclear factor-kB; 4-HNE, 4-hydroxynonenal.

PPARγ expression and transcriptional activity were dramatically reduced during the transdifferentiation process from a quiescent to an activated/fibrogenic myofibroblast-like phenotype [3,4]. Accordingly, synthetic PPARγ ligands, adenovirus-mediated ectopic expression of PPARγ or epigenetic PPARγ derepression achieved the phenotypic reversal from activated HSCs to the quiescent phenotype [4–6]. Consistent with these findings, administration of synthetic TZDs effectively exerted preventive actions of early inflammatory and fibrogenic response in different animal models of liver injury [7]. However, the therapeutic efficacy of TZDs appears to be limited and dependent on the type of injury, duration of disease and/or severity of fibrosis at the time of initiation of treatment [8]. Moreover, the pharmacological actions of TZDs seem to be also PPARγ-independent [9,10]. Overall, these studies indicate that the precise role of PPARγ in hepatic inflammation and fibrosis is still controversial.

In the current study, we further explored the role of PPARγ in liver inflammation and fibrosis by using the Cre-loxP system to specifically target individual liver cell types, namely hepatocytes, macrophages, represented in the liver as Kupffer cells, and HSCs. Our results reveal that specific disruption of PPARγ in macrophages and, to a lesser extent, in HSCs, exacerbates the inflammatory and fibrogenic response to carbon tetrachloride (CCl_4). The contribution of non-parenchymal cell PPARγ in controlling the hepatic inflammation-fibrosis axis was confirmed in lipopolysaccharide (LPS)-treated precision-cut liver slices (PCLS), which allows direct assessment of liver cells in the absence of extrahepatic and circulating factors. Considering that we recently identified PPARγ as a prosteatotic factor in parenchymal liver cells (i.e., hepatocytes) [11], the current findings demonstrating anti-inflammatory and anti-fibrogenic properties of PPARγ in non-parenchymal cells suggest the existence of divergent roles for hepatic PPARγ in the pathogenesis of liver injury.

Materials and methods

Mouse colonies

Because of their more distinguishable phenotype, the study was performed in heterozygous floxed/null PPARγ deficient mice harboring one loxP-targeted *PPARγ* allele (flanking exons 1 and 2) and a blunted *PPARγ* allele by the in-frame insertion of a LacZ-cassette (exon 2). These mice were crossed with transgenic mice expressing Cre recombinase under the control of either the albumin promoter (*Alb*-Cre), the myeloid-specific lysozyme M promoter (*LysM*-Cre) or the adipocyte fatty acid-binding protein 4 promoter (*aP2*-Cre) to generate mice with a specific targeted deletion of *PPARγ* in hepatocytes (*Alb*-Cre) (n = 16), macrophages (*LysM*-Cre) (n = 16) or HSCs (*aP2*-Cre) (n = 8), respectively [12–15]. These mice are predicted to loose PPARγ isoform 1 (PPARγ1) and a non-functional N-terminal, 43-aminoacid translational product of PPARγ isoform 2 (PPARγ2) that misses the partial AF1 domain and the first zinc finger of the DNA binding domain [13]. Heterozygous floxed/null mice were used as controls (CT). Mice breeding and DNA genotyping were performed as described in Supplementary Materials and methods.

Acute and chronic models of liver injury

Acute and chronic liver injury was induced as described in Supplementary Materials and methods. All animal studies were conducted in accordance with the criteria of the Investigation and Ethics Committee of the Hospital Clínic and the EU laws governing the use of experimental animals.

Histological analysis and detection of F4/80 by immunohistochemistry

Necroinflammatory injury, hepatic steatosis, hepatic fibrosis and the detection of F4/80, a specific marker of murine tissue macrophages, were assessed as described in Supplementary Materials and methods.

Hepatic hydroxyproline content

Hydroxyproline content was quantified as described in Supplementary Materials and methods.

Biochemical analysis

LDH, ALT, and AST levels were determined by standard laboratory procedures.

Isolation and culture of liver cells

Hepatocytes, Kupffer cells, and HSCs were isolated as described in Supplementary Materials and methods.

Isolation and culture of peritoneal macrophages and adipocytes

Resident peritoneal macrophages and adipocytes from wild type mice were isolated as described [16] (Supplementary Materials and methods).

PCLS

Organotypic cultures of liver slices from 22-week old CT (n = 8), *Alb*-Cre (n = 3) and *LysM*-Cre (n = 6) mice were performed as described [11] (Supplementary Materials and methods). Liver slices were pre-incubated at 37 °C in a 5% CO_2 incubator for 90 min before treatments and then incubated for 18 h with either vehicle ($DPBS^{-/-}$), LPS (100 μg/ml) (Sigma), rosiglitazone (10 μM) (Cayman Chemical, Ann Harbor, MI), and a combination of LPS and rosiglitazone. At the end of the incubation period, culture medium was collected to determine ALT and LDH levels and liver slices were snap-frozen in liquid nitrogen for gene expression analysis.

Analysis of gene expression

RNA concentration, integrity, and gene expression were assessed as described in Supplementary Materials and methods.

Immunocytochemistry of PPARγ in primary liver cells

Quiescent HSCs placed on Cytospin slides or activated HSCs cultured for 9 days in 8-chamber Polystyrene Vessel Tissue Culture Slides (BD Falcon) isolated from CT and *aP2*-Cre mice were fixed-permeabilized in ice-cold methanol:acetone (1:1) and stained with a primary rabbit anti-mouse PPARγ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as detailed in Supplementary Materials and methods. Kupffer cells were enriched by attachment for 1 h in Nunc Lab-Tek Chamber Slide system 8 wells Permanox Slide (Thermo Scientific, Waltham, MA), fixed, permeabilized, and immunostained as described above.

Analysis of PPARγ protein expression by Western blot

Nuclear extracts from mouse liver tissue were subjected to Western blot using primary rabbit anti-mouse PPARγ antibody (Santa Cruz Biotechnology) as detailed in Supplementary Materials and methods.

Analysis of cleaved-caspase-3 by Western blot

Total liver protein was extracted using a modified RIPA buffer and subjected to Western blot using primary rabbit anti-mouse cleaved-caspase-3 and caspase-3 antibodies (Cell Signaling, Danvers, MA) (Supplementary Materials and methods).