Survival and Inflammation Promotion Effect of PTPRO in Fulminant Hepatitis Is Associated with NF-κB Activation

Runqiu Jiang, Dianyu Chen, Jiajie Hou, Zhongming Tan, Youjing Wang, Xingxu Huang, Xuehao Wang and Beicheng Sun

*J Immunol* published online 22 October 2014
http://www.jimmunol.org/content/early/2014/10/19/jimmunol.1303354

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/10/19/jimmunol.1303354.4.DCSupplemental

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Survival and Inflammation Promotion Effect of PTPRO in Fulminant Hepatitis Is Associated with NF-κB Activation

Runqiu Jiang,*†,1 Dianyu Chen,*†,1 Jiajie Hou,*† Zhongming Tan,*† Youjing Wang,*† Xingxu Huang,‡ Xuehao Wang,*† and Beicheng Sun,*†

Previous investigations demonstrated that protein tyrosine phosphatase, receptor type, O (PTPRO) acts as a tumor suppressor in liver cancer; however, little is known about its role in liver inflammation. Thus, we investigated the role of PTPRO in fulminant hepatitis (FH) using a Con A–induced mouse model. Significantly more severe liver damage, but attenuated inflammation, was detected in PTPRO-knockout (KO) mice, and PTPRO deficiency could confer this phenotype to wild-type mice in bone marrow transplantation. Moreover, hepatocytes with PTPRO depletion were more sensitive to TNF-α–induced apoptosis, and secretion of cytokines was significantly decreased in both T and NK/NKT cells and led to marked impairment of NF-κB activation. Intriguingly, wild-type and PTPRO-KO cells responded equally to TNF-α in activation of IKK, but NF-κB activation was clearly decreased in PTPRO-KO cells. PTPRO associated with ErbB2, and loss of PTPRO potentiated activation of the ErbB2/Akt/GSK-3β/β-catenin cascade. Increased β-catenin formed a complex with NF-κB and attenuated its nuclear translocation and activation. Importantly, in humans, PTPRO was much decreased in FH, and this was associated with enhanced β-catenin accumulation but reduced IFN-γ secretion. Taken together, our study identified a novel PTPRO/ErbB2/Akt/GSK-3β/β-catenin/NF-κB axis in FH, which suggests that PTPRO may have therapeutic potential in this liver disease.

The Journal of Immunology, 2014, 193: 000–000.

F ulminant hepatitis (FH) is a rare, but potentially fatal, disease in which the patient’s condition rapidly deteriorates; it can cause hepatic encephalopathy, necrosis of the hepatic parenchyma, coagulopathy, renal failure, and coma. Without supportive management and/or liver transplantation, the mortality is ~70% (1). T cell–mediated liver damage is usually considered one of the principal pathological changes of FH. Hepatitis B or C viruses and autoimmune factors are the most common causes of lethal T cell–mediated liver damage in humans (2, 3). It is difficult to study FH using human samples because of its short duration and high mortality. Therefore, various animal models of T cell–mediated liver injury have been developed, including an FH model in mice that is induced by i.v. injection of the T cell–stimulatory plant lectin Con A (3).

Protein tyrosine phosphatase, receptor type, O (PTPRO) is an integral membrane protein that contains a single intracellular catalytic domain with a characteristic signature motif. There are several alternatively spliced variants that make up the tissue-specific isoforms of PTPRO (4). For example, hepatocytes contain the full-length PTPRO protein, whereas it appears as a truncated form (PTPROt) in T cells, which are some of the main infiltrating inflammatory cells during FH. Most studies of PTPRO’s role focused on cancer research; a common goal has been to define PTPRO as a tumor suppressor candidate (5–8). Very recently, we (5) reported that PTPRO inhibits development of hepatocellular carcinoma by suppression of JAK2/STAT3 signaling; however, its role in liver inflammation is largely unknown. In this study, we investigated the effect of PTPRO in both mouse and human FH. Our findings demonstrate that PTPRO is associated with the promotion of hepatocyte survival and proinflammatory effects by NF-κB activation via PTPRO/ErbB2/Akt/GSK-3β/β-catenin/NF-κB signaling.

Materials and Methods

Patients

Twenty-four patients who underwent liver transplantation because of FH at the First Affiliated Hospital of Nanjing Medical University from January 2009 to December 2012 were included in the study. Normal liver parenchymal tissues, used as controls, were obtained from 24 Chinese patients with benign disease, such as hemangioma. Informed consent for gene expression analysis was obtained from all patients prior to surgery. The study was approved by the institutional ethics committee of the First Affiliated Hospital of Nanjing Medical University in accordance with the Declaration of Helsinki.

Con A–induced liver injury model

Eight- to ten-week-old male C57BL/6 mice were purchased from the animal center of Nanjing Medical University. The PTPRO-knockout (KO) C57BL/6
mice were donated by Dr. John L. Bizby (University of Miami, Miami, FL) and were maintained and bred in the animal center of Nanjing Medical University. All mice were maintained under specific pathogen-free conditions in the animal facilities.

All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. In liver injury studies, mice received a Con A injection (20 μg/kg body weight) via the tail vein, as described previously (9). Detailed indices of liver injury were analyzed at 6, 9, 12, and 24 h after Con A injection.

Assay for serum transaminase activity
Serum samples from mice were obtained at different times. Serum alanine aminotransferase (ALT) activities were determined using the serum transaminase test kit (Rong Sheng, Shanghai, China), based on methods recommended by the International Federation of Clinical Chemistry.

Real-time PCR
Reverse-transcription reactions were performed using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). RTA templates were treated with DNase to avoid genomic DNA contamination. To quantify cDNA in the reverse-transcribed samples, real-time PCR analysis was performed using a 7300 Detection System (Applied Biosystems, Foster City, CA). Real-time PCR was performed according to the manufacturer’s instructions with a SYBR Premix Ex Taq kit (TaKaRa, Shiga, Japan). The primers used to detect various genes are provided in Table 1. Data were normalized to GAPDH levels in the samples.

Liver mononuclear cell preparation
Liver mononuclear cells (MNCs) were isolated as described (10). Briefly, mouse livers were removed and processed through a 100-μm cell strainer (BD), suspended in RPMI 1640 medium (Life Technologies, BRL), and centrifuged at 50 × g for 5 min. Supernatants containing hepatic MNCs were collected, washed in PBS, and resuspended in 40% Percoll (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 medium. The cell suspension was gently overlaid onto 70% Percoll and centrifuged for 20 min at 750 × g. MNCs were collected from the interphase and washed twice in PBS.

EMSA
For EMSAs, liver nuclear extracts were prepared, and the binding reaction was performed using a Light Shift Chemiluminescent EMSA Kit (Thermo Scientific, Waltham, IL), according to the manufacturer’s instructions. Free DNA and DNA protein complexes were resolved on a 6% polyacrylamide gel. A biotin-labeled oligonucleotide representing an NF-κB consensus site (5′-TAGTTAGGCGACCTTCCAGGCA-3′) was used as probe.

Hepatocyte isolation
Mouse livers were perfused in situ with 45 ml Life Technologies Liver Perfusion Media, followed by 45 ml Life Technologies Liver Digestion Media (both from Invitrogen). The liver digests were filtered through a cell strainer and washed with Gey’s Balanced Salt Solution (Sigma-Aldrich) containing Dnase I (2 mg/ml; Roche Diagnostics). The homogenate was centrifuged at 25 × g for 5 min at room temperature to obtain the hepatocytes. Isolated hepatocytes were washed thoroughly with PBS. After determination of cell viability (95%), hepatocytes were plated on 24-cm dishes and maintained in DMEM (Sigma-Aldrich) containing 10% FCS and a combination of penicillin and streptomycin for further analysis.

IKK activity assay
A total of 5 × 10⁶ hepatocytes was isolated from wild-type (WT) and PTPRO KO mice liver that was treated or not with TNF-α. Six hours later, the cells were lysed by incubation for 20 min at 4°C with RIPA plus a protease inhibitor mixture (Roche, Basel, Switzerland). The IKK complex was immunoprecipitated using Ab for IKKα and IKKβ (5 μg each). An enzymatic reaction was performed using 40 μl IKK complex, 2 μg GST-IXBox (1-45), and 0.5 μmol ATP and incubation at 30°C for 2 h. GST-IXBox was purified by GST Magnetic Beads (Promega, Madison, WI), size separated by SDS-PAGE, and probed by Western blotting with anti–p-IXBox (Abcam, Cambridge, MA).

TUNEL assay, H&E staining, and immunohistochemistry
The TUNEL test was performed with a TUNEL Apoptosis Detection Kit (Millipore), according to the manufacturer’s instructions, using a fluorescence microscope. H&E staining was performed using a standard protocol for cryosections. Immunohistochemistry was performed on paraffin-embedded sections, frozen sections, or isolated hepatocytes fixed on slides using Abs against TNF-α, IFN-γ, IL-6, PTPRO, p-IκBα, p-p65, p-ErbB2, and β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA).

ELISA
Mouse serum was collected and assessed using mouse epithelial growth factor (EGF) (Abcam) and TNF-α and IFN-γ ELISA kits (eBioscience). The experiments were performed in strict accordance with the instructions provided by the manufacturer.

Bone marrow transplantation
Six- to ten-week-old WT and PTPRO-KO mice were used as donors. Recipients were gamma irradiated with a cesium source (two 450-rad doses 4–5 h apart). A total of 1 × 10⁶ whole bone marrow cells isolated from the donor mice was transplanted into the recipients, and hematologic parameters were assessed at 8 wk posttransplant. Transplanted mice were maintained on 2.5 mg/100 ml Sulfatrim Pediatric Suspension (Alpharma, Baltimore, MD).

Coimmunoprecipitation and Western blot analysis
Proteins were extracted from mouse tissues and cells and quantitated using a protein assay (Bio-Rad, CA). Protein samples (30 μg) were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was conducted using Abs against PTPRO, p-p65 (S536), p65, p-IκBα (S32+S36), IκBα, XIAP, p-IκKα/β (S180+S181), total IKKα/β, p-ErbB2 (Y1248), ErbB2, p-β-catenin (Y654), β-catenin, p–GSK-3β (S9), GSK-3β, and TCF-1 (Santa Cruz Biotechnology). The results were visualized using a chemiluminescent detection system (Pierce ECL Western Blotting Substrate detection system; Thermo Scientific) and exposure to autoradiography film (Kodak XAR film).

TNF-α–induced apoptosis and PI–Annexin V containing
The isolated hepatocytes from WT or PTPRO-KO mice were treated with TNF-α (100 ng/ml) for various times and collected for apoptosis detection following the instructions for the PI–Annexin V staining kit (Invitrogen).

TaqMan low-density array and mouse phospho-receptor tyrosine kinase array
In order to detect the transcription of inflammatory related molecules, fresh liver tissues were obtained from the mice 12 h after Con A treatment. Reactions were carried out with an ABI 7900 HT thermal cycler using a TaqMan Array Mouse Immune Panel (4367786; both from Applied Biosystems). To screen for the differences in activity in receptor tyrosine kinases (RTKs) within TNFα–treated hepatocytes from WT and PTPRO-KO mice, protein was extracted and analyzed according to the instructions for the Mouse Phospho-Receptor Tyrosine Kinase Array (cat. no. ARY014; R&D Systems).

Statistical analysis
Results are expressed as mean ± SD. Comparisons between two groups were performed using the unpaired Student t test. Correlations between parameters were determined using Pearson correlation and linear regression analysis, as appropriate. All statistical analyses were performed using SPSS statistical software version 13.0, and two-tailed tests were applied to all data unless otherwise specified. A p value < 0.05 (95% confidence interval) was considered statistically significant.

Results
Increased liver injury and attenuated inflammation due to PTPRO depletion in Con A–induced mouse FH
Paired PTPRO-KO mice and littermate WT mice were injected with Con A. Significantly elevated serum ALT activity indicated that severe hepatic injury had occurred in PTPRO-KO mice (Fig. 1A). Liver damage was more severe in PTPRO-KO mice compared with WT mice; representative H&E results for both groups 24 h after Con A administration are shown in Fig. 1B1. Multiple necrosis and only a few integrated hepatic lobules were detected in livers of PTPRO-KO mice, and there also were dramatic differences between WT and PTPRO-KO mice at other time points (Supplemental Fig. 1A). Moreover, Suzuki’s criteria (10) were used to systematically evaluate the liver damage, which also indicated that significant serious injury had occurred as a result of
depletion of PTPRO (Fig. 1B2). Our findings showed that apoptosis correlated with increased liver injury, as detected by TUNEL assay. Significantly increased apoptosis in PTPRO-KO liver also was seen compared with WT liver at each time point (Fig. 1C1, 1C2). MNCs were isolated from mouse livers at 9 and 24 h following Con A injection. As controls, WT and PTPRO-KO mice were injected with PBS and sacrificed for MNC isolation. Inflammatory cell infiltration increased significantly in the livers of both WT and PTPRO-KO mice 9 and 24 h after Con A injection, but the numbers of MNCs decreased significantly in PTPRO KO mice compared with WT mice at these same time points (Fig. 1D). Furthermore, the expression levels of a set of inflammatory molecules were analyzed with low-density real-time PCR array using mRNA from total livers of KO and WT mice 12 h after Con A injection. The results were unexpected. As shown in Fig. 1E and Supplemental Fig. 1B, expression of proinflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, and IL-6, decreased in KO mice compared with WT mice, and the expression of chemokines, such as ccl2, ccl3, and cxc110, also decreased, which may explain the decrease in infiltrating cells in PTPRO-KO mice. Further investigations were performed to verify the differences among the three indices in which we were interested. The differences in TNF-α, IFN-γ, and IL-6 were confirmed by real-time PCR and immunohistochemistry, with both indicating significantly decreased secretion in PTPRO-KO mice (Supplemental Fig. 1C, 1D). Moreover, decreased expression of TNF-α, IFN-γ, and IL-6 in MNCs extracted from PTPRO KO mice compared with WT controls was observed by both real-time PCR and Western blot (Fig. 1F1, 1F2).

**NF-κB signaling of hepatocytes is decreased in PTPRO-KO mice**

Based on the cytokine-expression array results, we hypothesized that cell signaling also may affect the attenuated inflammation and decreased antiapoptotic abilities. Based on established knowledge about inflammation, particularly acute hepatitis, we investigated the status of NF-κB signaling in both groups.

To test this hypothesis, we first examined phosphorylation of NF-κBp65 (p-p65) and IκBα (p-IκBα), as well as expression of XIAP, an antiapoptotic protein that is one of the transcriptional products of NF-κB, in mouse livers. The levels of p-p65, p-IκBα, and XIAP were significantly decreased in PTPRO-deficient mice compared with WT controls (Fig. 2A, 2B, Supplemental Fig. 1E), suggesting that NF-κB activity was impaired in PTPRO-deficient mice. Furthermore, EMSA was used to assess NF-κB activation, and blockage of NF-κB activation in the liver of PTPRO-KO mice was confirmed (Fig. 2C).
To further study the blockage effect of NF-κB activation as a result of PTPRO depletion, hepatocytes were isolated and maintained in vitro. TNF-α–induced NF-κB activation was detected by immunofluorescence assay for p65 nuclear translocation (Fig. 2D1); nuclear translocation of p65 was quite evident, whereas it was blocked or attenuated in PTPRO-KO hepatocytes (Fig. 2D2). Meanwhile, apoptosis was detected by a PI–Annexin V staining assay. No spontaneous apoptosis was detected in WT or KO hepatocytes; however, significantly increased apoptosis was observed in PTPRO-KO hepatocytes (Fig. 2E, Supplementary Fig. 2A). Protein expression analysis by Western blot gave similar results; decreased p65 was detected in the nucleus, and decreased p-IκBα and XIAP were found in PTPRO-KO hepatocytes that underwent 6 or 24 h of TNF-α treatment. Surprisingly, the activation of IKKα/IκKβ, which are upstream kinases for NF-κB, was nearly intact in PTPRO hepatocytes compared with WT controls (Fig. 2F, Supplementary Fig. 2B), suggesting that an alternative pathway regulates NF-κB activation in PTPRO-KO hepatocytes.

**Functional impairment of T and NK/NKT cells occurs in PTPRO-KO mice as a result of inhibition of NF-κB activation**

Con A–induced hepatitis is characterized by massive hepatocellular degeneration and lymphoid infiltration of the liver; these depend primarily upon T and NK cell activation, which increases plasma levels of various cytokines, including IFN-γ and TNF-α. Liver MNCs were isolated from WT and KO mice, and cytokine secretion was determined by flow cytometry. MNCs were maintained in vitro and stimulated by Con A (10 μg/ml) for 24 h; decreased secretion of both IFN-γ and TNF-α was observed in T cells (Fig. 3A1, Supplementary Fig. 2C). Important subsets of hepatic inflammatory cells, NK/NKT cells, also were analyzed; significantly decreased cytokine secretion was observed, as well (Fig. 3A2, Supplementary Fig. 2D). CD4+ and CD8+ T cells were isolated from peripheral blood of WT and PTPRO-KO mice, and IFN-γ and TNF-α secretion was determined by ELISA with Con A treatment and controlled by PBS; secretion of both cytokines decreased significantly in PTPRO KO CD4+ and CD8+ T cells with Con A treatment (Fig. 3B1, 3B2). Next, CD4+ T cell subsets within MNCs were analyzed; there was no significant difference in the percentages of Th17 or regulatory T cells within liver MNCs after Con A treatment (Supplemental Fig. 3A). However, the percentage of Th1 cells was significantly decreased within the MNCs as a result of PTPRO depletion (Fig. 3C), which may have resulted from decreased IFN-γ secretion. To further study the mechanism of PTPRO, a truncated form of PTPRO, in hepatic inflammation (11, 12), T cells and NK/NKT cells were isolated from mouse spleen. In addition, the different forms of PTPRO, full-length PTPRO in hepatocytes and PTPROt in inflammatory cells, were confirmed by RT-PCR and Western blot (Table I, Supplementary Fig. 3B). ELISPOT was used to evaluate functional changes in PTPROt-depleted inflammatory cells. Significant decreases in both IFN-γ and TNF-α secretion were observed in PTPROt-depleted inflammatory cells. However, the percentage of Th1 cells was significantly decreased within the MNCs as a result of PTPRO depletion (Fig. 3C), which may have resulted from decreased IFN-γ secretion. To further study the mechanism of PTPRO, a truncated form of PTPRO, in hepatic inflammation (11, 12), T cells and NK/NKT cells were isolated from mouse spleen. In addition, the different forms of PTPRO, full-length PTPRO in hepatocytes and PTPROt in inflammatory cells, were confirmed by RT-PCR and Western blot (Table I, Supplementary Fig. 3B). ELISPOT was used to evaluate functional changes in PTPROt-depleted inflammatory cells. Significant decreases in both IFN-γ and TNF-α secretion were observed in PTPROt-depleted inflammatory cells.
TNF-α were found in PTPROt-depleted T and NK/NKT cells compared with WT cells (A1) and NK/NKT cells (A2) detected by flow cytometry. Comparison of the percentage of INF-γ and TNF-α T cells in PTPRO-KO and WT mice (n = 4/group). The secretion of INF-γ (B1) and TNF-α (B2) was determined by ELISA in the supernatant of CD4+ and CD8+ T cells treated with Con A (10 μg/ml) or PBS as controls in vitro. (C) Percentage of Th1 cells within MNCs isolated from mice 12 h after Con A treatment was analyzed by flow cytometry. T and NK/NKT cells were isolated from WT and PTPRO-KO mouse spleen (n = 4 for each group) and isolated using magnetic beads, and the secretion of INF-γ (D1) and TNF-α (D2) was evaluated by ELISPOT. (E) T and NK/NKT cells were isolated from mouse spleen using magnetic beads. Cells were collected 12 h following treatment with Con A (10 μg/ml), and protein extracts were used for Western blot analysis of p-p65, total p65, p-IKKα/β, and total IKKα/β. All data are mean ± SD. These results are representative of three independent experiments. **p < 0.01, unpaired Student t test.

PTPRO is associated with survival of hepatocytes and function of inflammatory cells

Based on the above results, we hypothesized that PTPRO might be associated with survival of hepatocytes and secretion of inflammatory cells.

Table I. Primer sequence for amplification of target genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’→3’)</th>
<th>Amplification Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>TCTTGAGGCCCCACTTTCTGCT</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>ACTCATCCTCCACCAGCTCTTGG</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>TGATTTTCTGGCCCTCTATTC</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>ACAGAGGGCCAAAAAGGTCT</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>AGGAAGCGGTCCAGGTAGTT</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>CTCCCAAGTCCTCCTCAATGACGAG</td>
<td></td>
</tr>
<tr>
<td>PTPRO/PTPROt</td>
<td>GAGGTGAAACCTTAAACCTGTT</td>
<td>511/427</td>
</tr>
<tr>
<td></td>
<td>TCAAGGAGACTAGGCTTCACCCC</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCCAGTGGTACAAGGCGAAAA</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>ACGCCAGTAGACCTCCACGACA</td>
<td></td>
</tr>
</tbody>
</table>
matory cells in the mouse model of FH. To test this hypothesis, bone marrow transplantation (BMT) was performed. As indicated in Fig. 4A, bone marrow samples from PTPRO-KO and WT mice were collected and transplanted into each other; the recipient mice were identified as BMT from WT mice to PTPRO-KO mice (W-P) and BMT from PTPRO KO mice to WT mice (P-W). WT BMT to WT was used as a control. First, the expression of PTPRO in peripheral MNCs was detected by Western blot analysis, indicating the success of BMT (Supplemental Fig. 3C). Next, the Con A–induced mouse FH model was set up; the three groups of mice were sacrificed for analysis 12 h following Con A administration. Serum ALT was assessed; the results revealed that W-P mice exhibited the most severe hepatic injury, whereas the livers of P-W mice exhibited the least damage (Fig. 4B). The histological results were in agreement with serum ALT analysis. Severe liver structural damage occurred in W-P mice, whereas there was almost no obvious damage in P-W mice (Fig. 4C); the difference in liver damage was reflected by Suzuki scores (Fig. 4D). The apoptosis of hepatocytes was evaluated by TUNEL assay, and the significance of the differences between W-P and the other two groups was assessed (Fig. 4E, Supplemental Fig. 3D). Without PTPRO, the hepatocytes were not exposed to the impact of cytokines, such as TNF-α; moreover, the function of inflammatory cells was restored after BMT. The degree of liver damage in W-P mice was even more marked in comparison with PTPRO-KO mice. In conclusion, PTPRO is associated with survival and function of inflammatory cells during mouse FH in association with NF-κB activation.

Accumulation of β-catenin induced by activation of ErbB2 inhibited NF-κB by direct binding due to PTPRO depletion

To identify a factor that is responsible for NF-κB suppression in PTPRO-deficient cells, a mouse RTK Ab array was used to detect TNF-α–treated hepatocytes isolated from both WT and PTPRO KO mice. We found that loss of PTPRO led to increased activation of several RTKs, including ErbB2, IGFr-1, Axl, Tie1, and Tie2 (Supplemental Fig. 4A). Among these activated RTKs, ErbB2 might serve as an ideal candidate because it can act either as a direct substrate of PTPRO or as an agonist for Wnt–β-catenin signaling (8, 13) and because EGF/ErbB2 signaling was involved in liver biology (14). Also, recent studies suggested that the accumulation of β-catenin can block activation of NF-κB via direct binding (15, 16). Thus, we reasoned that ErbB2 and β-catenin may mediate the suppression of NF-κB by PTPRO deficiency.

To test this scenario, the expression and activation of both ErbB2 and Wnt–β-catenin signaling were investigated in mouse liver...
markedly increased activation of ErbB2 and accumulation of β-catenin were verified in the livers of PTPRO-KO mice that were treated with Con A for 9 or 24 h. Moreover, phosphorylation of Akt and GSK-3β, as well as accumulation of their downstream targets β-catenin and TCF-1, was largely induced in PTPRO-deficient cells (Fig. 5A–C, Supplemental Fig. 4B). Furthermore, the hepatocytes were isolated and treated with TNF-α. Activation of Wnt–β-catenin in wild type hepatocytes was blocked, whereas nuclear translocation of p65 was quite obvious; in contrast, activation of Wnt–β-catenin in KO hepatocytes was significantly increased, and nuclear translocation of p65 was suppressed. Moreover, the inhibitors of Wnt–β-catenin and ErbB2 also were involved. When PTPRO-KO hepatocytes were treated with lapatinib, an universal inhibitor for the EGFR family, including ErbB2, decreased activation of Wnt–β-catenin and increased p65 nuclear translocation were observed. However, lapatinib cannot completely compensate for the suppression of NF-κB in PTPRO-KO hepatocytes, which might indicate that ErbB2 was just one of the RTKs promoting Wnt–β-catenin signaling. However, when Wnt–β-catenin was blocked by its inhibitor, FH535, nuclear translocation of p65 increased markedly, indicating that Wnt–β-catenin potentially plays a dominant role in PTPRO-regulated NF-κB suppression (Fig. 5D). Meanwhile, the functions of both T cells and NK/NKT cells were restored to a varying degree when treated with lapatinib and FH535 (Fig. 5E). In addition, the nuclear existence of p65 was analyzed in hepatocytes, T cells, and NK/NKT cells treated with TNF-α or Con A. Lapatinib only partially rescued p65 nuclear translocation, whereas FH535 restored it almost completely (Fig. 5F). Furthermore, coimmunoprecipitation (co-IP) assays were performed to confirm whether β-catenin can inhibit NF-κB by direct binding within hepatocytes. In PTPRO-KO hepatocytes, with or without TNF-α treatment (Fig. 5G1), β-catenin interacts with p65, which is in accordance with the outcome of previous research (15).

To determine whether PTPRO directly regulates ErbB2 activity, we first examined the serum level of EGF in both WT and PTPRO-

**FIGURE 5.** Accumulation of β-catenin induced by activation of ErbB2 inhibits NF-κB by direct binding due to PTPRO depletion. (A) Mice were treated with PBS or Con A, liver tissues were collected 9 and 24 h later (for Con A–treated mice only), and protein extracts were analyzed by Western blot for expression of p-ErbB2, total ErbB2, p–β-catenin, total β-catenin, GSK3β, TCF-1, p-AKT, total AKT, p-ERK1/2, and total ERK1/2. (B) Average integrated OD (IOD) for five fields for each slide was evaluated by Image-Pro Plus software (version 5.0) for immunohistochemical staining of p-ErbB2 and β-catenin. (C) T cells were isolated from mouse spleen using magnetic beads, the cells were treated with Con A (10 μg/ml) or PBS for 12 h, and protein extracts were analyzed by Western blot for expression of p-ErbB2, total ErbB2, p–β-catenin, total β-catenin, GSK3β, TCF-1, p-AKT, total AKT, p-ERK1/2, and total ERK1/2. (D) Hepatocytes were isolated from WT and PTPRO-KO mice (n = 4/group) and treated or not with TNF-α (100 ng/ml), lapatinib (100 μM), or FH535 (20 μM) (original magnification ×400). Immunofluorescence staining was performed 12 h later to detect p65 and β-catenin. Percentage of INF-γ and TNF-α–producing T cells (E1) and NK/NKT cells (E2) isolated from spleen of WT and PTPRO-KO mice following treatment with Con A (10 μg/ml) and lapatinib or FH535. (F) Expression of nuclear p65 was detected in TNF-α–treated (100 ng/ml) and Con A–treated (10 μg/ml) hepatocytes, T cells, and NK/NKT cells that were treated with lapatinib or FH535. (G1) In vivo co-IP assay in PTPRO-KO hepatocytes. Cell lysates from cells that were treated or not with TNF-α were incubated with anti–β-catenin or p65 Ab for immunoprecipitation. (G2) In vivo co-IP assay in WT hepatocytes. Cell lysates from cells were incubated with anti-ErbB2 Ab for immunoprecipitation. These results are representative of four independent experiments. All data are mean ± SD. *p < 0.05, **p < 0.01, unpaired Student t test.
deficient mice. Although the serum level of EGF was almost equal in WT and KO mice (Supplemental Fig. 4C), activation of ErbB2 by EGF clearly increased in PTPRO-deficient hepatocytes (Supplemental Fig. 4D). A further immunoprecipitation assay revealed that PTPRO was associated with ErbB2 in hepatocytes (Fig. 5G2), and our data confirmed previous results obtained from mammary epithelial cells that PTPRO can inhibit ErbB2 activation by direct binding (8).

Taken together, our data suggest that negative regulation of NF-κB by PTPRO is mediated by the ErbB2/Akt/Gsk-3β/β-catenin axis.

Low expression of PTPRO in hepatocytes and high expression of PTPRO in inflammatory cells may contribute to acute liver failure in human FH

To investigate the role of PTPRO in human FH, 24 patients who suffered from FH and had undergone liver transplantation were studied. Paraffin-embedded liver tissues were used for immunohistochemistry staining. The expression of PTPRO, p–ErbB-2, β-catenin, p-p65, and IFN-γ were detected and quantified by image software. Compared with normal liver tissues, the expression of IFN-γ, p-ErbB2, and β-catenin was significantly increased. Interestingly, there was almost no PTPRO expression in hepatocytes, whereas high expression levels of PTPRO were detected in nonparenchymal cells. Similarly to PTPRO, p-p65 was very low in hepatocytes, whereas strong staining was evident in inflammatory cells (Fig. 6A, 6B). After statistical analysis, we found that PTPRO was negatively correlated with the expression of β-catenin (Fig. 6C) and positively correlated with INF-γ expression (Fig. 6D) in the liver tissues of human FH. Therefore, our data suggested that low expression of PTPRO in hepatocytes and high expression of PTPRO in inflammatory cells may be associated with massive apoptosis and strong inflammation in human FH.

Discussion

Numerous studies suggested that PTPRO acts as a tumor suppressor because of its characteristic kinase inhibition. Low or aberrant expression of PTPRO has been verified in many kinds of human tumors, including lung cancer, hepatocellular carcinoma, colon cancer, and breast cancer (3, 4, 6, 17). A variety of its targets, which are involved in essential tumor-associated cell signaling, has been explored, and most of these also play an essential role in inflammation (18, 19). However, whether PTPRO is directly involved in regulating inflammatory responses is unclear.
Other protein tyrosine phosphatases were reported to play a regulatory role in inflammation (20). For example, loss of T cell protein tyrosine phosphatase expression results in synergists, with several hallmarks of inflammatory arthritis, within mouse femurs (21). In the current study, we demonstrated that PTPRO is a critical regulator in both human and mouse FH. Low or depleted expression of PTPRO in hepatocytes was associated with impaired cell function in both T and NK/NKT cells; secretion of IFN-γ, TNF-α, and other proinflammatory factors was associated with expression of PTPRO. A novel PTPRO/PTPROt/ErbB2/Akt/Gsk-3β/β-catenin/ NF-κB signaling pathway was identified within inflammatory cells and hepatocytes.

The strong increase in transaminase levels and more serious liver damage in PTPRO-KO mice suggested a protective role for PTPRO during Con A–induced acute liver injury. To test the functional relevance of PTPRO in hepatocytes, we stimulated them with TNF-α. Two pathways exist in hepatocytes following treatment with TNF-α: the survival pathway and the death pathway. The survival pathway is TRAF2-mediated, classic NF-κB–signaling activation that triggers transcription of a series of antiapoptosis genes, such as XIAP, Bcl-2, A-20, and c-Flip (22–24). As shown in this simple and feasible TNF-α–treated hepatocyte model, PTPRO is an essential factor that maintains the survival pathway. In contrast to WT control, TNF-α induces massive hepatocyte apoptosis in association with decreased NF-κB activation. As mentioned above, NF-κB is essential to trigger antiapoptotic gene expression in hepatocytes, which explains the lack of protection and increased apoptosis in PTPRO-KO mice. However, WT and PTPRO-deficient hepatocytes exhibited a similar activation of IKKα/IKKβ, whereas NF-κB activation was dramatically decreased in PTPRO-deficient hepatocytes, indicating that other factors are involved in regulating NF-κB activation.

Our results indicated that activation of NF-κB signaling was impaired in hepatocytes, as well as in inflammatory cells, such as T and NK cells, as a result of PTPRO or PTPROt depletion. Impairment of NF-κB in inflammatory cells could result in decreased secretion of cytokines that reflected as decreased inflammation, whereas impaired NF-κB activation in hepatocytes could decrease their antiapoptotic ability during the inflammation strike, which could result in increased liver damage. Therefore, more severe liver damage was observed in PTPRO-KO mice with Con A treatment, even when the inflammation was less severe compared with WT mice.

Indeed, using a mouse RTK array, we identified that ErbB2, a member of the EGF/ERBB family, was activated within liver with FH. It was suggested that amplification or overexpression of this gene has been shown to play an important role in the pathogenesis and progression of certain types of cancer (17, 25, 26). In breast cancer, ErbB2 was found to upregulate β-catenin via inactivation of GSK-3β by the Erk pathway (13). Also, in hepatocellular carcinoma, upregulation of ErbB-2 expression increased the existence of β-catenin (13, 27). Further, a study suggested that serum levels of ErbB2 might be associated with liver dysfunction (28). However, a role for ErbB2 in human liver disease is largely unclear. In the current study, we found that p-ErbB2 was mostly positive (75%) in human FH tissues and identified a PTPRO/Erbb2/Akt/β-catenin/NF-κB cascade (Fig. 6E).

β-Catenin is part of a complex of proteins that make up adherens junctions. Recent evidence suggested that β-catenin plays an important role in liver biology, including liver development, liver regeneration, and pathogenesis of liver cancer (29–31). Aside from its role in cancer, several studies focused on its role in acute liver injury; they all indicated that high expression of β-catenin can induce apoptosis through different mechanisms. Li et al. (32) reported that high levels of β-catenin in the HCC cell line can induce more rapid apoptosis compared with low β-catenin cells treated by IFN-γ as a result of p53 accumulation and Bcl-2 downregulation. Another study using a mouse hepatic ischemia-reperfusion model revealed that activation of β-catenin inhibited the TLR4–driven inflammatory response in dendritic cells by triggering PI3K/Akt signaling (33); the phenotype is similar to our model in which the accumulation of β-catenin in T or NK/NKT cells impairs their secreting function. Moreover, Zou et al. (16) demonstrated that the loss of β-catenin in hepatocytes significantly reduced their apoptosis in GalN/LPS–induced liver damage; its effect was attributed to inhibition of NF-κB signaling. Another study observed that the p65/β-catenin complex in hepatocytes undergoes dynamic changes during TNF-α–induced hepatic injury and plays a critical role in NF-κB activation and cell survival (15). The dynamic changes in p65/β-catenin complexes were impaired as a result of overactivated ErbB2 in PTPRO-depleted hepatocytes, which, in turn, activated Akt, but inactivated GSK-3β, leading to accumulation of β-catenin. Accumulated β-catenin formed a complex with NF-κB and blocked its nuclear translocation (Fig. 6E).

In summary, we identified that PTPRO is critical for FH development and that it negatively regulates NF-κB in FH via activation of the ErbB2/Akt/GSK-3β/β-catenin cascade. Our data suggest that PTPRO may be a therapeutic target for FH.

Disclosures
The authors have no financial conflicts of interest.

References


Figure S1.

A. ConA (25 mg/g body weight) was administered to C57BL/6 WT and PTPRO Knockout mice. H.E staining for mice liver 6, 9 and 12h after ConA administration. "N" represented necrosis area in mouse liver.

B. Fold change of relative mRNA expression compared to WT mice injected with PBS of various indices in the liver of both PTPRO KO and WT mice evaluated by low density real-time PCR array, all the indices were categorized into other cytokines, signaling and others.

C. Mouse liver tissues 12h after ConA injection were collected for real-time PCR and IHC detection. Average integrated optical density (IOD) was obtained by analyzing five fields for each slide evaluated by Image-Pro Plus software (version 5.0) for IHC staining of TNF-α, IFN-γ, and IL-6 (n=4 per group).

D. Representative figures for IHC staining of TNF-α, IFN-γ and IL-6 in ConA treated liver of WT and PTPRO KO mice.

E. Representative Figures for IHC staining of PTPRO, p-p65, p-IκBα and XIAP in ConA treated liver of WT and PTPRO KO mice.
Figure S2.

A. Hepatocyte was isolated from WT and PTPRO KO mice, and treated with TNF-α or PBS, cell apoptosis was accessed by PI-Annexin co-staining 6 (TNF-α treated only) and 24h after the treatment.

B. IKK activity assy. The WT and PTPRO KO mice were treated with ConA and PBS, 6h later, the protein was extracted, the activity of IKK complex was determined according to the methods described in the Experimental procedures section.

C and D. Liver MNCs were isolated from mice 12h after ConA injection, the secretion of INF-γ and TNFα in T and NK/NKT cells was detected by flow cytometry.
A1, A2. Liver MNCs were isolated from mice 12h after ConA injection, Comparison of the percentage of both Th17 and Treg in WT and PTPRO KO mice controlled by PBS. The determination was performed by flow cytometry using CD4 co-staining with IL-17A or Foxp3 respectively.

B1. The gene sequence of both PTPRO and PTPROT were aligned. A 78bp fragment which labeled in orange in PTPRO was obtained which only existed in PTPRO but not in PTPROT. Therefore, a pair of primers was designed to identify PTPRO and PTPROT expression with
different PCR product: 511bp for PTPRO and 427bp for PTPROt.

B2, B3. The mRNA and protein were obtained from 1. Liver from WT mice, 2. Hepatocyte isolated from WT mice, 3 T cells isolated form WT mice and 4.NK cells isolated form WT mice. The PCR amplification western-blot were performed.

C. Verification of PTPRO for BMT mice, Protein was extracted respective from hepatocyte and PBMCs of W-W, W-P and P-W mice.

D. TUNEL staining for apoptosis in mouse liver respectively from W-W, P-W and W-P group 12h after ConA injection.
Figure S4.

A. Mouse antibody array detection of hepatocytes treated with TNF-α isolated from PTPRO KO and WT mice.

B. IHC staining for p-ErbB2 and β-catenin. WT and PTPRO KO mice were treated with ConA, the liver was collected 12h after treatment, and the paraffin embedded tissues were analyzed by IHC staining for p-ErbB2 and β-catenin.

C. Serum EGF determination by ELISA. WT and PTPRO KO mice were treated with ConA or PBS, mice serum were collected 12h later, the serum concentration of EGF was determined by ELISA.

D. ErbB2 activation with the treatment of EGF in WT and PTPRO KO hepatocyte. The hepatocyte was isolated from both PTPRO KO and WT mice, the cells was treated with or without EGF (10ng/mL), 3h later, the cell lysate was analyzed by western-blot for ErbB2 (Y1248), total ErbB2, and α-Tubulin as a control.