Decreased hepatic peroxisome proliferator-activated receptor-γ contributes to increased sensitivity to endotoxin in obstructive jaundice

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AIM: To investigate the role of hepatic peroxisome proliferator-activated receptor-γ (PPAR-γ) in increased susceptibility to endotoxin-induced toxicity in rats with bile duct ligation during endotoxemia.

METHODS: Male Sprague-Dawley rats were subjected to bile duct ligation (BDL). Sham-operated animals served as controls. DNA binding were determined by polymerase chain reaction, Western blotting analysis, and electrophoretic mobility shift assay, respectively. BDL and sham-operated rats received a non-lethal dose of intraperitoneal lipopolysaccharide (LPS) injection (3 mg/kg, i.p.). Additionally, the potential beneficial effects of the PPAR-γ agonist rosiglitazone were determined in BDL and sham-operated rats treated with a non-lethal dose of LPS. Survival was assessed in BDL rats treated with a non-lethal dose of LPS and in sham-operated rats treated at a lethal dose of LPS (6 mg/kg, i.p.).

RESULTS: PPAR-γ activity in rats undergoing BDL was significantly lower than in the sham-controls. Hepatic PPAR-γ gene expression was downregulated at both the mRNA and protein levels. In a parallel group, serum levels of pro-inflammatory cytokines were nearly undetectable in the sham-operated rats. When challenged with a non-lethal dose of LPS (3 mg/kg), the BDL rats had approximately a 2.4-fold increase in serum IL-6, a 2.7 fold increase in serum TNF-α, 2.2-fold increase in serum IL-1 and 4.2-fold increase in serum ALT. The survival rate was significantly lower as compared with that in sham-operated group. Additionally, rosiglitazone significantly reduced the concentration of TNF-α, IL-1β, IL-6 and ALT in sham-operated rats, but not in BDL rats, in response to LPS (3 mg/kg). Also, the survival was improved by rosiglitazone in sham-operated rats challenged with a lethal dose of LPS, but not in BDL rats, even with a non-lethal dose of LPS (6 mg/kg).

CONCLUSION: Obstructive jaundice downregulates hepatic PPAR-γ expression, which in turn may contribute to hypersensitivity towards endotoxin.

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also speculated that the PPAR-γ expression and function in the liver are decreased by endotoxemia and/ or increased proinflammatory cytokines during obstructive jaundice. Here we examined whether hepatic PPAR-γ expression and function are decreased in a rat model of obstructive jaundice, to seek for a possible correlation between alteration of PPAR-γ expression and increased susceptibility to endotoxin.

MATERIALS AND METHODS

Animals
Male Sprague-Dawley rats (250-300 g) were purchased from the Animal Center of Shanghai Jiao Tong University School of Medicine (Shanghai, China) and were housed in an air-filtered room at 22-25 °C on a 12-h light/dark cycle, with unlimited access to water and standard rat chow. All experimental procedures were in accordance with the institutional animal care guidelines and approved by the local ethic committees.

Bile duct ligation
Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The common bile duct was ligated and divided after laparotomy. Sham-operated rats underwent the same procedure without bile duct ligation.

Experimental protocol
Three sets of experiments were carried out. In the first experiment, rats were sacrificed at day 7 after the surgery to analyze PPAR-γ expression and activation in the liver. In the second experiment, endotoxemia was established by a non-lethal dose of lipopolysaccharide (LPS) (Escherichia coli 0111:B4; Sigma, 3 mg/kg, i.p.) in the BDL or sham-operated rats. Blood was collected for analysis of pro-inflammatory cytokines (e.g., TNF-α, IL-6 and IL-1β) and alamine aminotransferase (ALT) 2 h after LPS challenge. Survival was also monitored in another group. In the third experiment, the potential beneficial effects of the PPAR-γ agonist rosiglitazone were examined in BDL and sham-operated rats treated with a non-lethal dose of LPS (3 mg/kg, i.p.). Survival was examined in BDL rats treated with a non-lethal dose of LPS and in sham-operated rats treated at a lethal dose of LPS (6 mg/kg, i.p.). Rosiglitazone (3 mg/kg, i.p.) or vehicle (10% dimethyl sulfoxide) was administered intraperitoneally as a bolus 15 min prior to LPS injection.

RNA extraction and peroxisome proliferator-activated receptor-γ gene expression
Total RNA was extracted from hepatic tissues using TRIzol reagent (Invitrogen, United States) according to the manufacturer’s protocol. Primers were obtained from Shanghai Sangon Biologic Engineering and Technology and Service (Shanghai, China). The following primer sequences were used: for PPAR-γ mRNA (forward: 5'-ACACCATGCTGGCCTCCTGTA-3'; reverse: 5'-AAGGCTGGGCGG-GTCTCCACT-3'; size 220 bp), and for β-actin (forward: 5'-CCACACCCCGACACACTGCG-3'; reverse: 5'-CTTCGCTTGGCGTCGC-3'; size 205 bp). Amplifica...
tion and detection were performed with an ABI PRISM 7300 real-time polymerase chain reaction (PCR) System (Applied Biosystems, Foster City, California, United States) as follows: 30 s at 95 °C, and 40 cycles at 95 °C for 5 s and at 60 °C for 31 s. The DNA-binding dye SYBER Green I for the detection of PCR products was used. The reaction mixture (RT-PCR kit, Code DRR063A, Takara) contained 25 μL Premix Ex Taq, 1 μL forward and reverse primers, 1 μL ROX reference dye, 4 μL cDNA (equivalent to 20 ng total RNA) in a final volume of 50 μL. The amount of gene transcript was measured using a comparative (2^{-ΔΔCT}) method by Applied Biosystems. The reference gene β-actin was used for normalization of the expression data.

### Western blotting analysis of peroxisome proliferator-activated receptor-γ proteins

The nuclear extracts were prepared from liver tissues using a nuclear extract kit (Active Motif, Carlsbad, CA), following the manufacturer’s protocol. Protein concentration was measured by the bicinchoninic acid assay method (Pierce). Liver nuclear extracts containing equal amounts of protein were separated in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE). Amounts of protein were separated in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE). The reaction mixture (RT-PCR kit, Code DRR063A, Takara) contained 25 μL Premix Ex Taq, 1 μL forward and reverse primers, 1 μL ROX reference dye, 4 μL cDNA (equivalent to 20 ng total RNA) in a final volume of 50 μL. The amount of gene transcript was measured using a comparative (2^{-ΔΔCT}) method by Applied Biosystems. The reference gene β-actin was used for normalization of the expression data.

### Electrophoretic mobility shift assay of peroxisome proliferator-activated receptor-γ activation

Electrophoretic mobility shift assay (EMSA) was used to detect specific binding of the transcription factor PPAR-γ to its specific DNA consensus sequence. Nuclear protein/DNA binding reactions were conducted for 20 min at room temperature in a 20-μL reaction volume containing 2 μL 10 × binding buffer, 1 μL poly(dC)-dC, 1 μL 50% glycerol, 1 μL 1% NP-40, 1 μL of 1 mol/L KCl, 1 μL 100 mmol/L MgCl2, 1 μL 200 mmol/L EDTA (all the reagents are included in the LightShift Chemiluminescent EMSA kit; Pierce), 20 fmol biotin-labeled probe (PPAR-γ consensus sequence 5’-GGGTCATAGTCACTGGAGGCCAGGGA-3’), and 2 μL nuclear extract. Binding reactions were analyzed using 8% PAGE. After blotting to a nylon membrane, the labeled oligonucleotides were detected with the LightShift Chemiluminescent EMSA kit (Pierce). The relative intensity of the bands was analyzed using an LAS-1000 luminoimage analyzer (Fujifilm, Tokyo, Japan).

### Measurement of serum cytokines and alanine aminotransferase

Serum concentrations of TNF-α, IL-1β, or IL-6 were quantified using enzyme-linked immunosorbent assay (R&D Systems). Serum ALT levels were determined with an autotmizer (Model 7600, Hitachi Co., Tokyo, Japan).

### Statistical analysis

The data were expressed as means ± SE. Data were analyzed by analysis of variance, followed by the Student-Newman-Keuls test. The survival curve was estimated by the Kaplan-Meier method and statistical significance was assessed by log-rank test. P values less than 0.05 were considered as significant.

### RESULTS

#### Peroxisome proliferator-activated receptor-γ activation and expression are decreased upon bile duct ligation-induced cholestasis

We started the investigation by measuring modification of PPAR-γ activation and expression upon BDL and consequent cholestasis. We measured levels of PPAR-γ activation through electrophoretic mobility shift assay of PPAR-γ DNA binding in nuclear extracts from liver of treated and control rats (Figure 1A). PPAR-γ activity in rats undergoing BDL was significantly lower than in the sham-controls (0.62 ± 0.07 vs 1.00 ± 0.08, P = 0.0257). Hepatic PPAR-γ gene expression was downregulated at both the mRNA (1 ± 0.09 vs 0.09 ± 0.03, P = 0.0000002, Figure 1B) and protein levels (0.70 ± 0.02 vs 0.59 ± 0.01, P = 0.0089, Figure 1C).

#### Enhanced susceptibility toward endotoxin-induced toxicity in rats with pre-existing cholestasis

In a parallel group, serum levels of pro-inflammatory cytokines were nearly undetectable in the sham-operated rats. Biliary obstruction per se resulted in the induction of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 (66.0 ± 6.5 vs 27.9 ± 3.2, P = 0.00036; 56.0 ± 7.6 vs 19.7 ± 2.6, P = 0.0011; 60.2 ± 5.8 vs 30.7 ± 3.5, P = 0.0014, Table 1), and liver injury, as indicated by increased circulating level of ALT (119.7 ± 11.1 vs 41.2 ± 3.2, P = 0.00005, Table 1). When challenged with a non-lethal dose of LPS (3 mg/kg), the BDL rats had approximately 2.75-fold increase in serum TNF-α, 2.21-fold increase in serum IL-1β, 2.41-fold increase in serum IL-6 and 4.17-fold increase in serum ALT (2364.7 ± 196.3 vs 861.1 ± 44.2, P = 0.00006; 2373.0 ± 263.1 vs 1073 ± 97.7, P = 0.00098; 10802.0 ± 853.2 vs 4476.7 ± 430.2, P = 0.00005; 305.0 ± 37.5 vs 73.2 ± 7.7, P = 0.0001, Table 1). The survival rate was significantly lower than in the sham-operated group (20% vs 90%, P = 0.000009, Figure 2). These results indicated that enhanced susceptibility toward endotoxin-induced toxicity is present in BDL rats, which is consistent with previous studies.

#### Peroxisome proliferator-activated receptor-γ agonist rosiglitazone does not protect jaundiced rats from endotoxemia

To further verify a possible correlation between alterations of PPAR-γ function and increased susceptibility...
to endotoxin, we investigated whether PPAR-γ agonist rosiglitazone had anti-inflammatory effects in BDL rats. Treatment with rosiglitazone significantly reduced the concentration of TNF-α, IL-1β, IL-6, and ALT in sham-operated rats, but not in BDL rats, in response to LPS (3 mg/kg) (Table 2). The survival was improved by rosiglitazone in sham-operated rats challenged with a lethal dose of LPS (6 mg/kg) (50% vs 20%, P = 0.049) but not in BDL rats, even with a non-lethal dose of LPS (35% vs 25%, P = 0.49) (Figure 3). These data indicated that endogenous anti-inflammatory pathway of nuclear receptor PPAR-γ is impaired by obstructive jaundice, which in turn may be at least in part, responsible for the increased susceptibility to endotoxin.

**DISCUSSION**

In this study, both the expression and function of PPAR-γ in the liver were significantly depressed in rats with BDL as compared with the sham-operated rats. Concomitant to the decrease in PPAR-γ DNA binding, we observed a markedly increased susceptibility to endotoxin, evidenced by higher degree of liver injury, enhanced proinflammatory cytokine release, and a higher mortality. The PPAR-γ
agronist rosiglitazone failed to protect BDL rats against endotoxaemia.

Despite the use of broad-spectrum antibiotics and improvement in surgical technique, patients with cholestatic liver disease continue to experience a high incidence of postoperative morbidity and mortality\cite{29,30}. Belghiti et al\cite{31} showed that the mortality of patients with obstructive jaundice is much higher (21%) than that of the patients with normal liver function. Among the multiple complications from obstructive jaundice, gram-negative bacterial sepsis is the most common cause of secondary morbidity and mortality\cite{31,32}. Animal studies have demonstrated that obstructive jaundice exaggerates the release of several cytokines after endotoxin administration, including TNF-α, IL-1β, and IL-6\cite{8-11}.

KCs are the largest macrophage population and form the first line of defense against microorganisms entering the portal circulation. It has been demonstrated that the KCs are the primary source of circulating TNF-α and IL-6 in response to LPS\cite{24}. In the context of appropriate immune response, KC activation plays a protective role during the acute phase of hepatopathies. However, in response to prolonged bacterial or endotoxin challenge, KC over-activation may increase the severity of organ damage and lethality. With regards to obstructive jaundice, previous studies indicate that liver KCs are involved in the exaggerated cytokine secretion after endotoxin challenge\cite{11,24}. In jaundiced animals undergoing endotoxin challenge, blockade of KC function with gadolinium chloride or TNF-α antibody has been shown to improve survival and to suppress the systemic proinflammatory response\cite{14}. Consequently, altered function of KCs during cholestasis is thought to play a decisive role in the increased susceptibility to endotoxin-induced toxicity.

PPARs are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily. Recently, it has been found that PPAR-γ activation inhibits the expression of several inflammatory response genes in activated macrophages, including the genes encoding inducible nitric oxide synthase, TNF-α, gelatinase B, and COX-2\cite{24,25}. Moreover, activation of PPAR-γ reduces the organ injury/dysfunction caused by endotoxin\cite{24} and by hemorrage and resuscitation\cite{26}, as well as systemic inflammation caused by zymosan\cite{27} and by cecal ligation and puncture\cite{28} in rodents. In this study, the expression and function of hepatic PPAR-γ were decreased during obstructive jaundice. PPAR-γ agonist rosiglitazone failed to protect BDL rats from lethal endotoxaemia, suggesting that the downregulation of PPAR-γ in jaundiced rats is of functional significance.

It should be pointed out that hepatic PPAR-γ is expressed in both KCs and hepatocytes (HCs). We did not examine the source of PPAR-γ activity in this study. Nevertheless, previous studies have indicated that PPAR-γ gene expression decreased significantly in KCs at 20 h after sepsis by cecal ligation and puncture, whereas PPAR-γ expression in HCs was not altered. Moreover, when isolated KCs or HCs from normal rats were stimulated with LPS or TNF-α for 20 h, KC PPAR-γ protein levels were all significantly decreased. In contrast, neither LPS nor TNF-α affects PPAR-γ protein levels in HCs either in monoculture or in co-culture with KCs. These findings suggest that the KCs and HCs respond to LPS and TNF-α differentially\cite{17}. Therefore, we believe that the downregulated PPAR-γ expression in the liver during obstructive jaundice is most likely due to decreased PPAR-γ in KCs but not in HCs.

Recent studies have indicated the potential efficacy of PPAR-γ ligands as novel therapeutic approaches in sepsis, inflammation and reperfusion injury. However, our observation that treatment with a PPAR-γ ligand fails to provide protection in cholestatic animals suggests that activation of the endogenous PPAR-γ pathway may need to be tailored to the specific conditions, e.g., obstructive jaundice.

Evidence suggests that the PPAR-γ expression is a function of the inflammatory response. For instance, in rats subjected to sepsis by cecal ligation and puncture or...
double-hit hemorrhage and sepsis, PPAR-γ expression is downregulated in the liver, bronchial epithelium, and vascular endothelium\[13,15\]. In mice subjected to endotoxin administration, PPAR-γ expression is also markedly reduced in adipose tissues, heart, and lungs\[16-30\]. Consistent with these reports, the current study demonstrated that biliary obstructive jaundice significantly increases serum amounts of TNF-α, IL-6 and IL-1β. Thus, increased proinflammatory cytokines (e.g., TNF-α) during cholestasis may result in downregulation of the PPAR-γ in the liver.

In conclusion, biliary obstructive jaundice downregulates hepatic PPAR-γ expression and function, which in turn may contribute to enhanced susceptibility to endotoxin-induced toxicity.

**COMMENTS**

**Background**

Biliary obstructive jaundice increases susceptibility to endotoxin-induced toxicity. However, the underlying molecular mechanisms are not fully understood.

**Research frontiers**

 Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the nuclear receptor family of ligand-activated transcription factors. Activation of PPAR-γ could produce anti-inflammatory effects. In this study, the authors demonstrated that hepatic PPAR-γ is down-regulated upon obstructive jaundice, and provided some evidence suggesting that the down-regulation of PPAR-γ contributes to the hypersensitivity to endotoxin.

**Innovations and breakthroughs**

The expression and function of hepatic PPAR-γ were significantly decreased in the bile duct ligation rats compared with the control rats. Down-regulation of PPAR-γ was accompanied by exaggerated inflammatory response. Treatment with the PPAR-γ agonist rosiglitazone protected sham-operated, but not BDL rats, from endotoxemia.

**Applications**

Recent studies have suggested the potential efficacy of PPAR-γ ligands as novel therapeutic approaches in sepsis, inflammation, and reperfusion injury. The treatment with a PPAR-γ ligand fails to provide protection of cholestatic animals suggests that activation of the endogenous PPAR-γ pathway needs to be tailored to the specific conditions, e.g., obstructive jaundice.

**Peer review**

This is an elegant study from a biological observation to a potential clinical aspect.

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