

## Research Article

# Peroxisome Proliferator-Activated Receptor Gamma Exacerbates Concanavalin A-Induced Liver Injury via Suppressing the Translocation of NF- $\kappa$ B into the Nucleus

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Peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) has been reported to reduce inflammation and attenuate fibrosis in the liver. In this study, we investigated the effects of PPAR $\gamma$  on the liver injury induced by 20 mg/kg Concanavalin A (Con A). The mice were administered one of the three types of PPAR $\gamma$  ligands (pioglitazone, ciglitazone, and troglitazone) for 1 week, and the serum alanine aminotransferase (ALT) levels at 20 h after Con A injection were significantly elevated in the PPAR $\gamma$  ligand-treated mice. Furthermore, the serum ALT levels after Con A injection in the PPAR $\gamma$  hetero-knock-out mice (PPAR $\gamma^{+/-}$  mice) were lower than those in the wild-type mice (WT mice). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) revealed extensive liver damage induced by Con A in the pioglitazone-treated mice. Electrophoresis mobility shift assay (EMSA) revealed that activation of translocation of nuclear factor- (NF-)  $\kappa$ B, which is a suppressor of apoptosis, in the nucleus of the hepatocytes was suppressed in the pioglitazone-treated mice after Con A injection. In this study, we showed that PPAR $\gamma$  exacerbated Con A-induced liver injury via suppressing the translocation of NF- $\kappa$ B into the nucleus, thereby inhibiting the suppression of liver cell apoptosis.

## 1. Introduction

PPARs are members of the nuclear receptor superfamily [1]. Three isotypes designated PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$  have been described in mammals [2]. The PPARs form heterodimers with the retinoid X receptor (RXR), and the PPAR-RXR heterodimers, when bound to a ligand, change their conformation and bind to the DNA at the PPAR response elements, which results in gene transcription [3, 4]. PPAR $\gamma$  is expressed in adipose tissue, heart, kidney, skeletal muscle, liver and other organs PPAR $\gamma$  ligands improve insulin resistance and inflammation by increasing serum adiponectin levels [5–7]. Thus, thiazolidinediones (TZDs), which are PPAR $\gamma$  ligands, are widely used in the treatment of type 2 diabetes mellitus (DM).

Liver injury is caused by various factors such as viral infections, autoimmune reactions, and metabolic disorders. Recently, PPAR $\gamma$  agonists have received attention in relation to the treatment of liver diseases. PPAR $\gamma$  has been reported to reduce hepatic inflammation by decreasing the expression of tumor necrosis factor $\alpha$  (TNF- $\alpha$ ) [8], and suppressing the translocation of NF- $\kappa$ B into the nucleus [9]. Furthermore, the PPAR pathway inhibits the fibrogenic actions in hepatic stellate cells and attenuates liver fibrosis *in vivo* [10, 11]. PPAR $\gamma$  agonists have been reported to be useful in mice and humans with NAFLD [12–14], as PPAR $\gamma$  promotes adipocyte differentiation [15], increases triglyceride storage in adipocyte, and reduces delivery of fatty acids to the liver [9]. However the effect on other liver diseases has not yet been investigated.

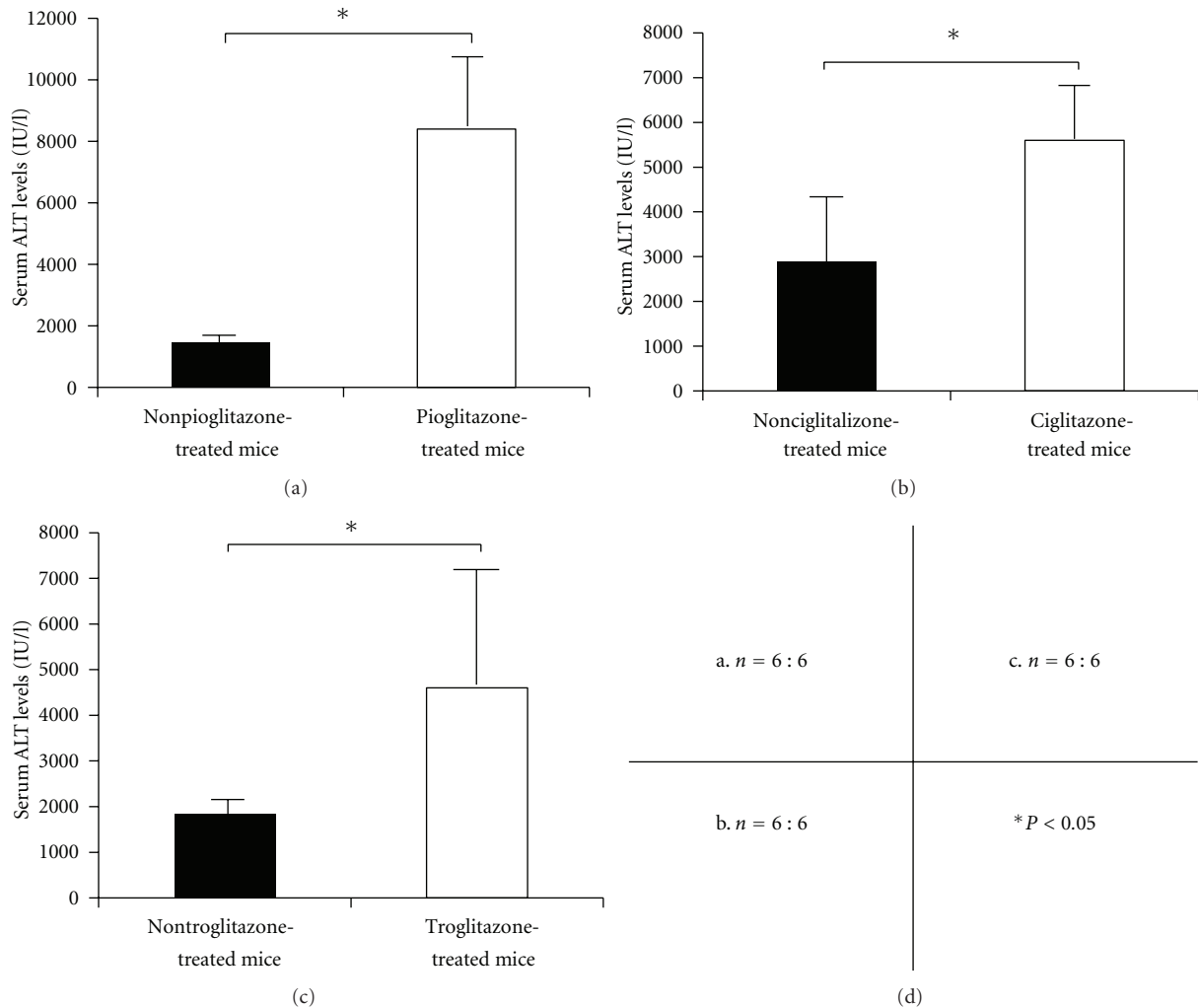


FIGURE 1: At 20 h after Con A injection, the serum ALT levels in the mice treated with one of the three types of PPAR $\gamma$  ligands (pioglitazone (a), troglitazone (b), ciglitazone (c)) were significantly higher as compared with those in the non-PPAR $\gamma$ -treated mice ( $*P < 0.05$ ).

In this study, PPAR $\gamma$  ligands and PPAR $\gamma^{+/-}$  mice were used to confirm the effects of PPAR $\gamma$  on the liver injury induced by Con A. Con A induces serious hepatitis in mice by activating T cells and triggering apoptosis [16, 17].

## 2. Materials and Methods

**2.1. Animal Experiments.** Eight-week-old male WT BALB/c mice and eight-week-old male PPAR $\gamma^{+/-}$  mice on a BALB/c background were purchased from CLEA Japan, Inc. and Jackson Laboratory (Bar Harbor, ME, USA), respectively. All the mice were maintained in filter-topped cages on autoclaved normal chow diet containing 22% protein, 6% fat, and 47% carbohydrate. In the Con A-induced hepatitis model, Con A (Sigma Aldrich, St. Louis, MO, USA; 20 mg/kg) was injected intravenously (i.v.) into mice. First, WT mice ( $n = 6$  mice) were fed either a control chow or chow supplemented with one of the two types of PPAR $\gamma$  ligands (ciglitazone (100 mg/kg) and troglitazone (150 mg/kg)) *ad libitum* for 1 week and sacrificed at 20 h after the Con A injection.

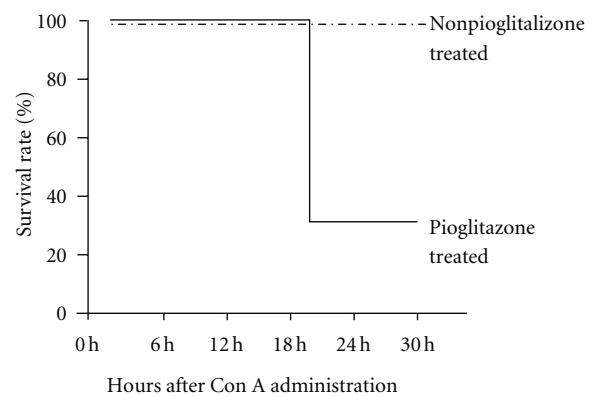


FIGURE 2: At 20 h after Con A injection, there were no cases of fatality in the nonpioglitazone-treated group of mice, whereas the fatality rate was 70% in the pioglitazone-treated mice.

These doses and duration of treatment with the PPAR $\gamma$  agonists were selected based on the efficacy demonstrated in

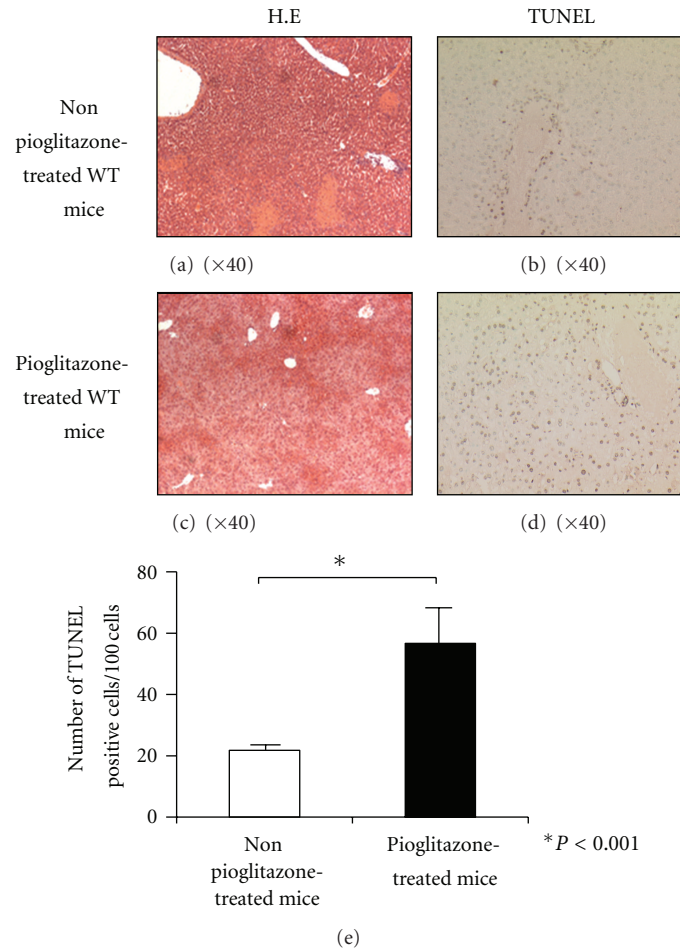


FIGURE 3: Histopathological examination of liver sections stained with H-E at 20 h after Con A injection revealed more extensive liver necrosis in the pioglitazone-treated mice (c) in comparison with that in the nonpioglitazone-treated mice (a). TUNEL assay at 20 h after Con A injection revealed more extensive liver apoptosis in the pioglitazone-treated mice (d) as compared with that in the nonpioglitazone-treated mice (b). The number of TUNEL-positive cells/100 cells was three-times higher in the livers of the pioglitazone-treated mice as compared with that in the livers of the nonpioglitazone-treated mice ( $56 \pm 8.01$  versus  $21 \pm 1.83$ ,  $P < 0.001$ ).

pilot experiments (data not shown). Subsequently, PPAR $\gamma^{+/-}$  mice were treated with either control chow or pioglitazone-supplemented chow and sacrificed at 20 h after the Con A injection. Finally, to investigate the effect of PPAR $\gamma$  on the activation of NF- $\kappa$ B induced by Con A, control or pioglitazone-supplemented chow was administered to the WT mice and sacrificed at various time points (0.5 h, 1 h, 3 h, 6 h, and 8 h) after the Con A injection, to obtain nuclear protein. The animal protocols were approved by the Yokohama City University Medical School Guidelines for the Care and Use of Laboratory Animals.

**2.2. Biochemistry.** Serum alanine aminotransferase (ALT) levels were measured by a local laboratory for clinical examinations (SRL Co, Ltd., Tokyo, Japan).

**2.3. Liver Histology.** Liver specimens were fixed overnight in buffered formaldehyde (10%) and embedded in paraffin.

Paraffin sections were prepared at 5  $\mu$ m thickness and stained with hematoxylin and eosin (H-E).

**2.4. Assay for Apoptosis.** The apoptotic tumor cells were stained using a TUNEL staining kit, according to the manufacturer's instructions (Wako Pure Chemical, Osaka, Japan). In brief, paraffin sections were digested with 20  $\mu$ g/mL of proteinase K (Takara, Shiga, Japan) for 15 min at room temperature and reacted with terminal deoxynucleotidyl transferase enzyme for 60 min at 37°C. The sections were then incubated with antidigoxigenin conjugate at room temperature for 30 min, followed by incubation with diaminobenzidine solution.

**2.5. Electrophoretic Mobility Shift Assay (EMSA).** NF- $\kappa$ B binding was determined by EMSA. We collected liver tissue specimens at various time points (0.5, 1, 3, 6, and 8 h) after the Con A injection. Nuclear protein extracts (10  $\mu$ g) were prepared using Nuclear Extraction kit (BizScience, Osaka,

Japan), according to the manufacturer's instructions. The probe oligonucleotide was 22 bp, double-stranded (5'-GCCTGGGAAAGTCCCCTCAACT-3') and end-labeled with biotin (Sigma Chemical, St. Louis, MO). DNA-protein complexes were resolved at 80 V for 1 h in a taurine-buffered, native 6% polyacrylamide gel (4% for supershift) and blotted onto a positively charged nylon membrane (Sigma Chemical, St. Louis, MO). Transferred DNA was immediately cross-linked to the membrane on an ultraviolet transilluminator equipped with 312 nm bulbs and detected using horseradish peroxidase-conjugated streptavidin (Light-Shift Chemiluminescent EMSA kit), according to the manufacturer's instructions.

**2.6. Statistical Analysis.** Data are presented as means  $\pm$  SD. Differences between the two groups were assessed using the unpaired two-tailed Student's *t*-test; *P* values of  $<0.05$  were considered to denote significance. All statistical analyses were performed using Microsoft Excel and the SPSS 16.0 statistical package (SPSS, Chicago, IL)

### 3. Results and Discussion

To assess the degree of liver injury, we analyzed the time course of changes of the serum ALT levels after the Con A injection. Unexpectedly, the serum ALT levels in the pioglitazone- (30 mg/kg) treated mice were significantly higher in comparison with that in the nonpioglitazone-treated mice at 20 h after Con A injection (Figure 1(a)). The survival rate of the nonpioglitazone-treated mice was 100%, while that of the pioglitazone- (30 mg/kg) treated mice was 30% at 20 h after Con A injection (Figure 2). Subsequently, we conducted a histological examination to assess the degree of Con A-induced liver injury at 20 h after Con A injection in the mice treated and not treated with 30 mg/kg of pioglitazone. Histopathological examination of tissue sections stained with H-E revealed that the liver damage was more extensive in the pioglitazone-treated mice as compared with that in the nonpioglitazone-treated mice (Figures 3(a) and 3(c)). To determine the presence and extent of apoptotic cells, we performed TUNEL assay. More TUNEL-positive hepatocytes could be detected in the liver sections of the pioglitazone-treated mice than in those of the control mice (Figures 3(b) and 3(d)). The number of TUNEL positive cells/100 cells in the livers of the pioglitazone-treated mice was three-times higher as compared with that in the livers of the nonpioglitazone-treated mice ( $56.2 \pm 8.0$  versus  $21.0 \pm 1.8$ ,  $P < 0.001$ ). From these results, we hypothesized that PPAR $\gamma$  might actually exacerbate Con A-induced liver injury by intensifying hepatocyte apoptosis. Then, we used two other PPAR $\gamma$  ligands (ciglitazone and troglitazone) to confirm the effect of PPAR $\gamma$ . All of the three PPAR $\gamma$  ligands produced a significant increase of the serum ALT levels in the treated mice as compared with the levels in the untreated mice (Figures 1(a), 1(b), and 1(c)). This result indicates that PPAR $\gamma$  ligands exacerbate Con A-induced liver injury regardless of the kinds.

To evaluate the effect of PPAR $\gamma$  on liver injury, we used PPAR $\gamma^{+/-}$  mice. The reason for using the heterologous

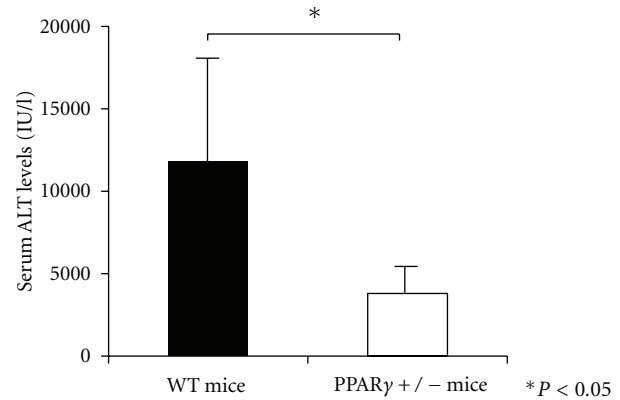


FIGURE 4: At 20 h after Con A injection, the serum ALT levels in the WT mice were significantly higher than those in the PPAR $\gamma^{+/-}$  mice.

PPAR $\gamma^{+/-}$  mice was that double knock-out of this gene results in embryonic lethality. Con A-induced liver injury was less extensive in the PPAR $\gamma^{+/-}$  mice as compared with that in the WT mice (Figure 4). This result suggests the possible involvement of the endogenous PPAR $\gamma$ -mediated pathway in the exacerbation of Con A-induced liver injury.

To confirm the apoptosis in the pioglitazone-treated and nonpioglitazone-treated mice after Con A-injection, we analyzed the expression of NF- $\kappa$ B, which is a known suppressor of apoptosis. To determine the quantity of activated NF- $\kappa$ B, we performed EMSA. Activation of NF- $\kappa$ B in the hepatocyte nuclei after Con A-injection was suppressed in the livers of the pioglitazone-treated mice as compared with that in the livers of the nonpioglitazone-treated mice (Figure 5). This result suggests that PPAR $\gamma$  suppresses the translocation of NF- $\kappa$ B into the nucleus, thereby inhibiting the suppression of liver cell apoptosis. Maeda et al. reported that hepatocyte-specific IKK $\beta$  knockout mice exhibit little NF- $\kappa$ B activity and are highly susceptible to liver apoptosis of Con A-induced liver injury [18].

From this study, suppression of PPAR $\gamma$ , such as using PPAR $\gamma$  antagonists may potentially reduce the extent of liver injury.

### 4. Conclusion

In this study, we showed that PPAR $\gamma$  ligands exacerbate Con A-induced liver injury via suppressing the translocation of NF- $\kappa$ B into the nucleus. Con A-induced liver injury in PPAR $\gamma$ -treated mice represents an intensified apoptosis. PPAR $\gamma$  antagonists may be considered as novel candidates for the therapy of liver injury in an intensifying apoptosis model.

### Abbreviations

PPAR $\gamma$ :	Peroxisome proliferator-activated receptor- $\gamma$
NAFLD:	Nonalcoholic fatty liver disease
Con A:	Concanavalin A
ALT:	Alanine aminotransferase
WT mice:	Wild-type mice

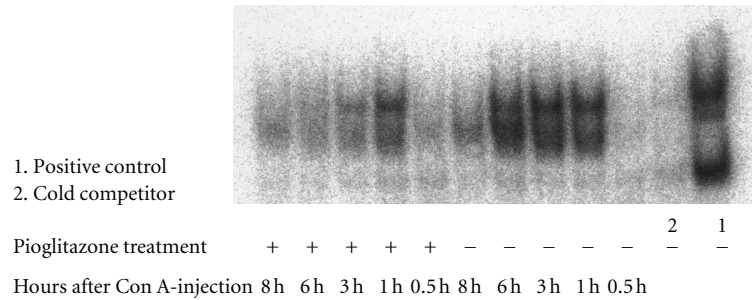


FIGURE 5: NF- $\kappa$ B binding was determined by EMSA. We collected liver tissue specimens at various time points (0.5, 1, 3, 6, and 8 h) after Con A injection. At 1 h after the Con A injection, NF- $\kappa$ B was activated in the nonpioglitazone-treated mice, whereas the NF- $\kappa$ B activation was suppressed in the pioglitazone-treated mice.

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling  
 EMSA: Electrophoresis mobility shift assay  
 NF: Nuclear factor  
 RXR: Retinoid X receptor  
 TZDs: Thiazolidinediones  
 DM: Diabetes mellitus  
 TNF- $\alpha$ : Tumor necrosis factor  $\alpha$   
 H-E: Hematoxylin and eosin.

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