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Research Article

Peroxisome Proliferator-Activated Receptor Gamma Exacerbates Concanavalin A-Induced Liver Injury via Suppressing the Translocation of NF-κB into the Nucleus

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Peroxisome proliferator-activated receptor-γ (PPARγ) has been reported to reduce inflammation and attenuate fibrosis in the liver. In this study, we investigated the effects of PPARγ on the liver injury induced by 20 mg/kg Concanavalin A (Con A). The mice were administered one of the three types of PPARγ 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 PPARγ ligand-treated mice. Furthermore, the serum ALT levels after Con A injection in the PPARγ 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-) κB, 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 PPARγ exacerbated Con A-induced liver injury via suppressing the translocation of NF-κB 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 PPARα, PPARβ/δ, and PPARγ 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]. PPARγ is expressed in adipose tissue, heart, kidney, skeletal muscle, liver and other organs PPARγ ligands improve insulin resistance and inflammation by increasing serum adiponectin levels [5–7]. Thus, thiazolidinediones (TZDs), which are PPARγ 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, PPARγ agonists have received attention in relation to the treatment of liver diseases. PPARγ has been reported to reduce hepatic inflammation by decreasing the expression of tumor necrosis factorα (TNF-α) [8], and suppressing the translocation of NF-κB into the nucleus [9]. Furthermore, the PPAR pathway inhibits the fibrogenic actions in hepatic stellate cells and attenuates liver fibrosis *in vivo* [10, 11]. PPARγ agonists have been reported to be useful in mice and humans with NAFLD [12–14], as PPARγ 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.

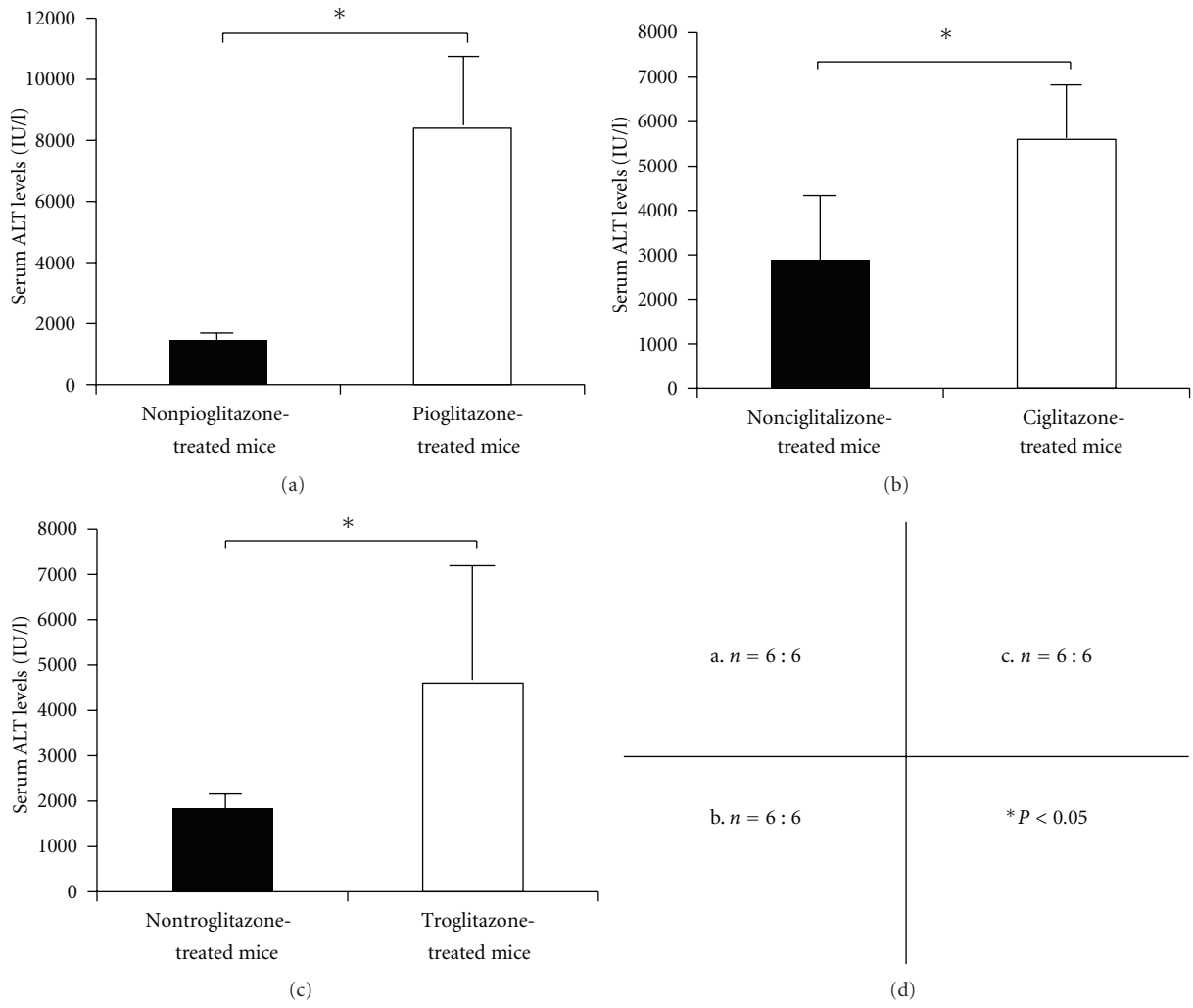

Figure 1: At 20 h after Con A injection, the serum ALT levels in the mice treated with one of the three types of PPARγ ligands (pioglitazone (a), troglitazone (b), ciglitazone (c)) were significantly higher as compared with those in the non-PPARγ-treated mice ($^*P < 0.05$).

In this study, PPARγ ligands and PPAR$\gamma^{+/-}$ mice were used to confirm the effects of PPARγ 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 PPARγ 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.

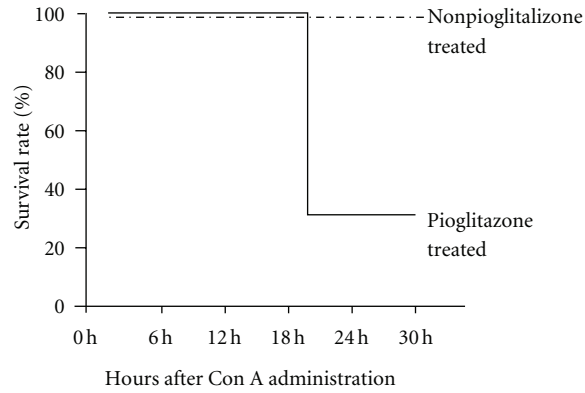

Figure 2: At 20 h after Con A injection, there were no cases of fatality in the nonpioglitazone-treated group of mice, whereas the fatality rate was 70% in the pioglitazone-treated mice.

These doses and duration of treatment with the PPARγ agonists were selected based on the efficacy demonstrated in

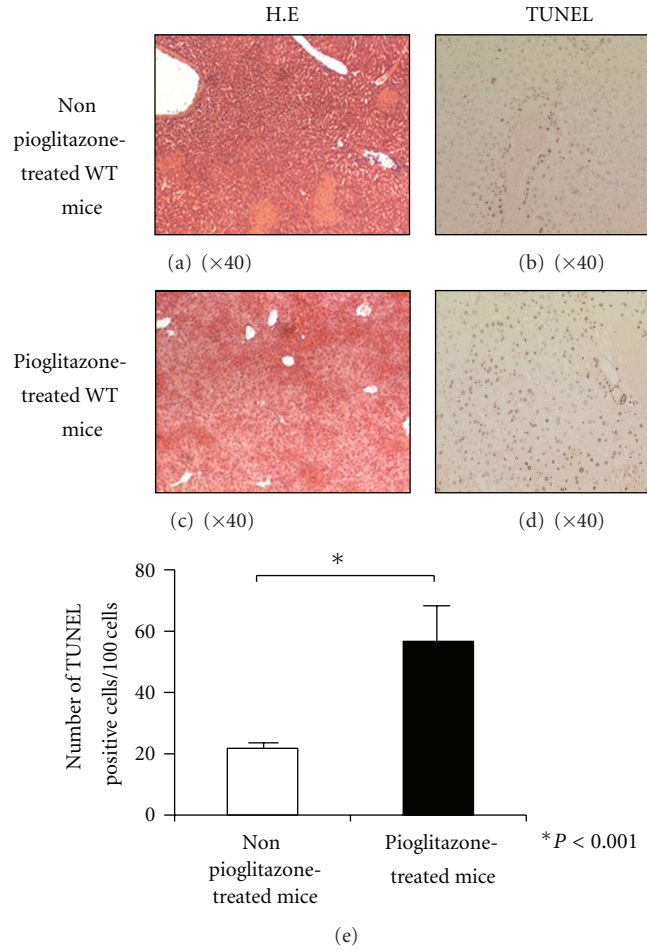

Figure 3: Histopathological examination of liver sections stained with H-E at 20 h after Con A injection revealed more extensive liver necrosis in the pioglitazone-treated mice (c) in comparison with that in the nonpioglitazone-treated mice (a). TUNEL assay at 20 h after Con A injection revealed more 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 ± 8.01 versus 21 ± 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 PPARγ on the activation of NF-κB 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 μm 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 μg/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-κB 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 μg) 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 endlabeled 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 ± 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 ± 8.0 versus 21.0 ± 1.8, $P < 0.001$). From these results, we hypothesized that PPARγ might actually exacerbate Con A-induced liver injury by intensifying hepatocyte apoptosis. Then, we used two other PPARγ ligands (ciglitazone and troglitazone) to confirm the effect of PPARγ. All of the three PPARγ 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 PPARγ ligands exacerbate Con A-induced liver injury regardless of the kinds.

To evaluate the effect of PPARγ on liver injury, we used PPAR$\gamma^{+/-}$ mice. The reason for using the heterologous 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 PPARγ-mediated pathway in the exacerbation of Con A-induced liver injury.

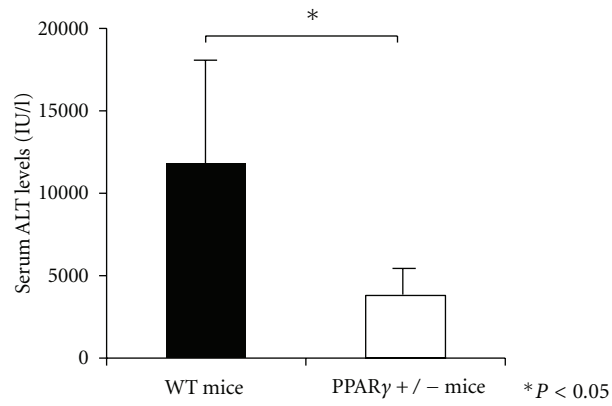

Figure 4: At 20 h after Con A injection, the serum ALT levels in the WT mice were significantly higher than those in the PPAR$\gamma^{+/-}$ mice.

To confirm the apoptosis in the pioglitazone-treated and nonpioglitazone-treated mice after Con A-injection, we analyzed the expression of NF-κB, which is a known suppressor of apoptosis. To determine the quantity of activated NF-κB, we performed EMSA. Activation of NF-κB 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 PPARγ suppresses the translocation of NF-κB into the nucleus, thereby inhibiting the suppression of liver cell apoptosis. Maeda et al. reported that hepatocyte-specific IKKβ knockout mice exhibit little NF-κB activity and are highly susceptible to liver apoptosis of Con A-induced liver injury [18].

From this study, suppression of PPARγ, such as using PPARγ antagonists may potentially reduce the extent of liver injury.

4. Conclusion

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

Abbreviations

PPARγ: Peroxisome proliferator-activated receptor-γ
NAFLD: Nonalcoholic fatty liver disease
Con A: Concanavalin A
ALT: Alanine aminotransferase
WT mice: Wild-type mice

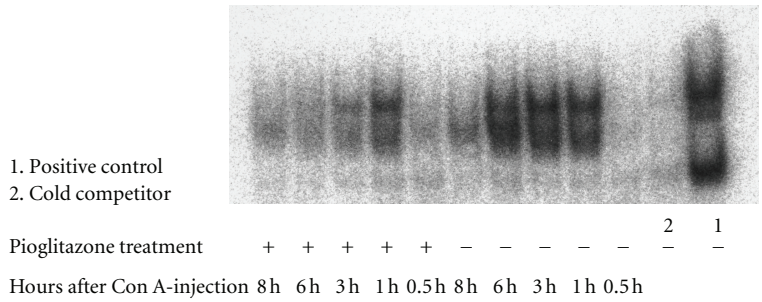

Figure 5: NF-κB binding was determined by EMSA. We collected liver tissue specimens at various time points (0.5, 1, 3, 6, and 8 h) after Con A injection. At 1 h after the Con A injection, NF-κB was activated in the nonpioglitazone-treated mice, whereas the NF-κB activation was suppressed in the pioglitazone-treated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

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

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