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Pioglitazone Prevents Early-Phase Hepatic Fibrogenesis Caused by Carbon Tetrachloride

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Here we investigated the effect of pioglitazone, a peroxisome proliferator-activated receptor (PPAR)-γ ligand, on early-phase hepatic fibrogenesis *in vivo* caused by acute carbon tetrachloride (CCl_4) administration in the rat. Pioglitazone (1 mg/kg BW) prevented pericentral fibrosis and induction of α-smooth muscle actin (SMA) 72 h after CCl_4 administration (1 ml/kg BW). CCl_4 induction of α1(I)procollagen mRNA in the liver was blunted by pioglitazone to the levels almost 2/3 of CCl_4 alone. Pioglitazone also prevented CCl_4-induced hepatic inflammation and necrosis, as well as increases in serum tumor necrosis factor-α levels. Further, pioglitazone inhibited the induction of αSMA and type I collagen in primary cultured hepatic stellate cells in a dose-dependent manner. In conclusion, pioglitazone inhibits both hepatic inflammation and activation of hepatic stellate cells, thereby ameliorating early-phase fibrogenesis in the liver following acute CCl_4. © 2002 Elsevier Science (USA)

Key Words: **peroxysome proliferator-activated receptor (PPAR); thiazolidinedione derivatives; pioglitazone; hepatic fibrogenesis; carbon tetrachloride; hepatic stellate cells; type I collagen; tumor necrosis factor (TNF)-α.**

Pioglitazone, a thiazolidinedione derivative (TZD), is an insulin-sensitizing drug clinically used for the treatment of type 2 diabetes patients worldwide (1). Like other TZDs (i.e., troglitazone, rosiglitazone and ciglitazone), pioglitazone decreases plasma glucose levels by reducing insulin resistance (2). TZDs are known as the specific ligand for peroxysome proliferator activated receptor (PPAR)γ (3). To date, three isotypes of PPARs, α, β/δ, and γ, have been identified as members of nuclear receptor superfamily of ligand-activated transcription factors (4). PPARs form a heterodimer with the retinoid X receptor (RXR), the receptor for 9-*cis*-retinoic acids, and these complex regulate the gene expression by binding to PPAR responsive element (PPRE) (5). PPARγ is expressed predominantly in adipose tissues, and it plays a pivotal role in the differentiation of adipocytes and metabolism of fatty acid (6). The prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 ($15dPGJ_2$) is the most potent natural ligand of PPARγ (7). TZDs are believed to exert their diverse pharmacological actions mainly through interaction with PPARγ, though existence of alternative pathways not involving PPARγ has also been postulated (4).

Hepatic fibrogenesis is a fundamental, clinically serious problem in various types of chronic liver diseases. Hepatic stellate cells (HSCs) are believed to play a pivotal role in fibrogenesis in the liver (8). Recently, HSCs have been shown to express PPARγ1, a general-type isoform of PPARγ (9). Further, it has been demonstrated that $15dPGJ_2$ and TZDs prevent proliferation, transactivation and collagen synthesis in isolated HSCs (9–11). In the present study, therefore, we investigated whether pioglitazone, a PPARγ ligand, prevents early hepatic fibrogenesis *in vivo* induced by acute carbon tetrachloride (CCl_4) administration in the rat. Preliminary accounts of this study have appeared elsewhere as an abstract form (12).

MATERIALS AND METHODS

Animal experiments. Specific pathogen-free female Wistar rats weighing 210–290 g (Charles River Japan Inc., Saitama, Japan) were injected CCl_4 [1 ml/kg body weight (BW), 1:1 in olive oil, Wako Chemical Inc., Osaka, Japan] and/or pioglitazone [AD-4833(HCl), 1 mg/kg BW, dissolved in dimethyl sulfoxide and suspended in olive oil, a generous gift from Takeda Chemical Industries Ltd., Osaka, Japan] intraperitoneally. Subsequently, the same amount of pioglitazone, or vehicle alone as controls, was injected 24 and 48 h later. Animals were sacrificed 12, 24, and 72 h after CCl_4 injection by exsanguination from inferior vena cava under light ether anesthesia. All animals received humane care and the experimental protocol was approved by the Committee of Lab-

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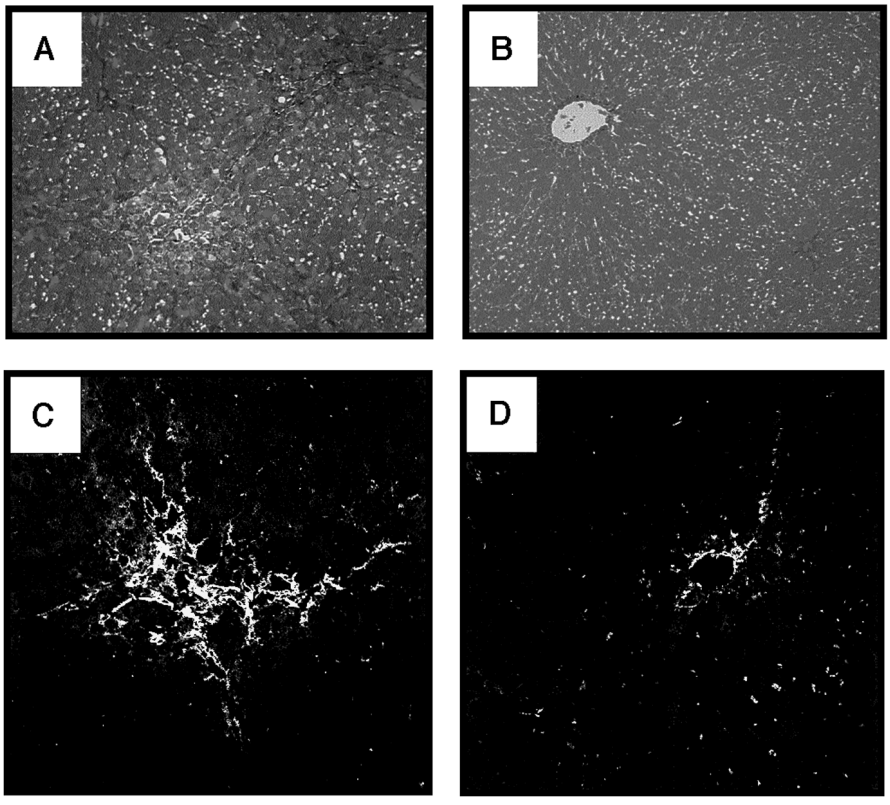

FIG. 1. Effect of pioglitazone on liver histology and expression of αSMA following acute carbon tetrachloride (CCl_4) administration. Rats were given intraperitoneal injections of CCl_4 (1 ml/kg BW) and/or pioglitazone (1 mg/kg BW) simultaneously. Subsequently, the same doses of pioglitazone or vehicle (olive oil) alone as controls were injected every 24 h, and liver samples were obtained 72 h later. (A and B) Liver histology. Representative photomicrographs of liver histology from CCl_4 treatment alone (A) and combination of CCl_4 and pioglitazone (B) are shown (Azan staining, original magnification: ×100). (C and D) Immunohistochemistry for αSMA. The expression and localization of αSMA in the liver were detected by immunohistochemical staining using a monoclonal antibody for αSMA. Representative photomicrographs from CCl_4 treatment alone (C) and combination of CCl_4 and pioglitazone (D) are shown (original magnification: ×200).

oratory Animals according to institutional guidelines. Hematoxylin–eosin (H-E) and Azan staining were performed, and serum and tissue samples were kept frozen at −80°C until assayed.

Measurement of serum transaminases and tumor necrosis factor (TNF)-α levels. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured spectrophotometrically using a commercial kit (KAINOS Laboratories Inc., Tokyo, Japan). Serum TNF-α levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine M, R&D Systems Inc., Minneapolis, MN).

Isolation and primary culture of hepatic stellate cells (HSCs). Hepatic stellate cells (HSCs) were isolated from female Wistar rats by *in situ* collagenase perfusion and differential centrifugation using Metrizamide (Sigma Chemical Co., St. Louis, MO) density gradients as previously described elsewhere (13). Isolated HSCs were inoculated on type-I collagen-coated polystyrene dishes (Sumitomo Bakelite Co., Tokyo, Japan) and cultured in Dulbecco's modified essential medium (DMEM, Sigma Chemical Co.) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin sulfate) in humidified air containing 5% CO_2 at 37°C for up to 7 days.

Immunohisto(cyto-)chemical staining for α-smooth muscle actin (αSMA) and type I collagen. For immunohisto(cyto-)chemical staining of α-smooth muscle actin (SMA), deparaffinized tissue sections or ethanol-fixed HSCs were incubated with a primary, mouse monoclonal anti-SMA antibody (American Research Products, Inc., Belmont, MA) and a secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology), and photomicrographs were taken using a fluorescence microscope (Axiophot ZVS3C75DE, Carl Zeiss Inc., Goleta, CA).

To detect type-I collagen in cultured HSCs, immunocytochemical staining was performed using a primary goat anti-type I collagen antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) and horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Santa Cruz Biotechnology). The specific staining was visualized using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), and photographed under a phase contrast microscope (Eclipse E600, Nikon, Tokyo, Japan).

Western blotting for αSMA. Whole cell protein extracts were prepared as previously described (14), and 5 μg of protein was analyzed by Western blotting using an anti-SMA antibody as previously described elsewhere (13) with slight modifications. Specific bands were visualized using the ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

RNA preparation and RNase protection assay. Total RNA was prepared from frozen livers by a guanidium/cesium trifluoroacetate (CsTFA) centrifugation method using QuickPrep total RNA extraction kit (Amersham Pharmacia Biotech). The concentration and purity of isolated RNA were determined by the optical density at 260 and 280 nm. RNase protection assay (RPA) was performed using MAXIscript *in vitro* transcription kit and RPA III kit (Ambion Inc., Austin, TX) as previously described elsewhere (15). The reaction was

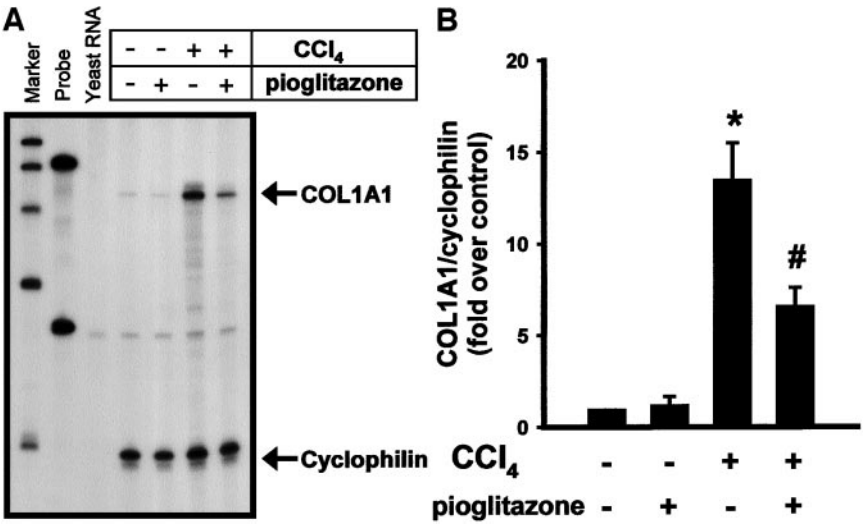

FIG. 2. Effect of pioglitazone on α1(I)procollagen mRNA in the liver following acute CCl_4. Experimental design as in Fig. 1. Steady-state mRNA levels for α1(I)procollagen in the liver 72 h after CCl_4 and/or pioglitazone treatment were measured using RNase protection assay. A representative photograph of protected bands simultaneously detected for α1(I)procollagen (COL1A1, 426 bp) and cyclophilin (103 bp), a housekeeping gene, from 4 individual experiments is shown (A). Yeast RNA was used as a negative control. The ratio of densitometrical values for COL1A1 and cyclophilin (expressed as fold over controls) was plotted (B). Data represent means ± SEM ($n = 4$, $^*P < 0.05$ vs controls, $\#P < 0.05$ vs CCl_4 alone, by ANOVA and Tukey's post-hoc test).

separated on a denaturing 5% polyacrylamide/urea gel and exposed to X-ray films (X-Omat AR, Kodak) at −80°C. Densitometric analysis was performed using Scion Image (Scion Corp., Frederick, MD).

Statistical analysis. Data were expressed as means ± SEM. Statistical differences between means were determined using analysis of variance (ANOVA) or ANOVA on ranks, followed by Tukey's or Dunn's multiple comparison procedure, as appropriate. $P < 0.05$ was selected prior to experiments to reflect statistical significance.

RESULTS

Effect of Piogliazone on Fibrotic Changes in the Liver Following Acute CCl_4 Administration

To investigate the effect of pioglitazone on hepatic fibrogenesis *in vivo,* rats were given a single injection of carbon tetrachloride (CCl_4, 1 ml/kg BW, i.p.) in combination with repeated injections of pioglitazone (1 mg/kg BW, i.p.) every 24 h for 3 days. Figure 1 demonstrates representative photomicrographs of liver histology by Azan staining. Acute CCl_4 administration caused mild pericentral fibrosis 72 h later (Fig. 1A). In sharp contrast, this fibrotic change caused by acute CCl_4 administration was prevented almost completely when pioglitazone was given every 24 h (Fig. 1B). Repeated injections of pioglitazone alone for 3 days caused no histological changes in the liver (data not shown).

Next, the expression of αSMA, a marker of activated HSCs, in the liver 72 h after CCl_4 administration was detected by immunohistochemical staining (Figs. 1C and 1D). As expected, specific staining for αSMA was observed intensely in pericentral area of the liver 72 h after a single injection of CCl_4 (Fig. 1C). This CCl_4-induced expression of αSMA in the liver was suppressed dramatically by repeated injections of pioglitazone (Fig. 1D). Livers from rats given pioglitazone alone, as well as vehicle controls, showed negative staining for αSMA as expected (data not shown).

Further, the steady-state mRNA levels of α1(I) procollagen, a component of type I collagen, were detected by RNase protection assay (Fig. 2). As expected, α1(I)-procollagen mRNA in the liver was increased about 3-fold 72 h after CCl_4 injection; however, pioglitazone prevented this increase to the levels nearly 1/3 of CCl_4 alone. Collectively, these findings indicated that pioglitazone prevents early profibrogenic responses in the liver following acute CCl_4 administration.

Effect of Pioglitazone on Necroinflammatory Responses in the Liver Following Acute CCl_4 Administration

Since PPARγ is postulated to be involved in macrophage differentiation and inflammatory responses (16–19), we next addressed the question whether pioglitazone prevents necroinflammatory responses in the liver following acute CCl_4 intoxication. As expected, massive necrosis in pericentral and mid-zonal area with infiltration of inflammatory cells was observed in the liver 24 h following acute CCl_4 administration (Fig. 3A). Coadministration of pioglitazone prevented this CCl_4-induced necroinflammatory change in the liver largely (Fig. 3B). Indeed, coadministration of pioglitazone prevented increases in serum AST and ALT levels 24 h after CCl_4 (Figs. 3C and 3D). Pioglitazone per se

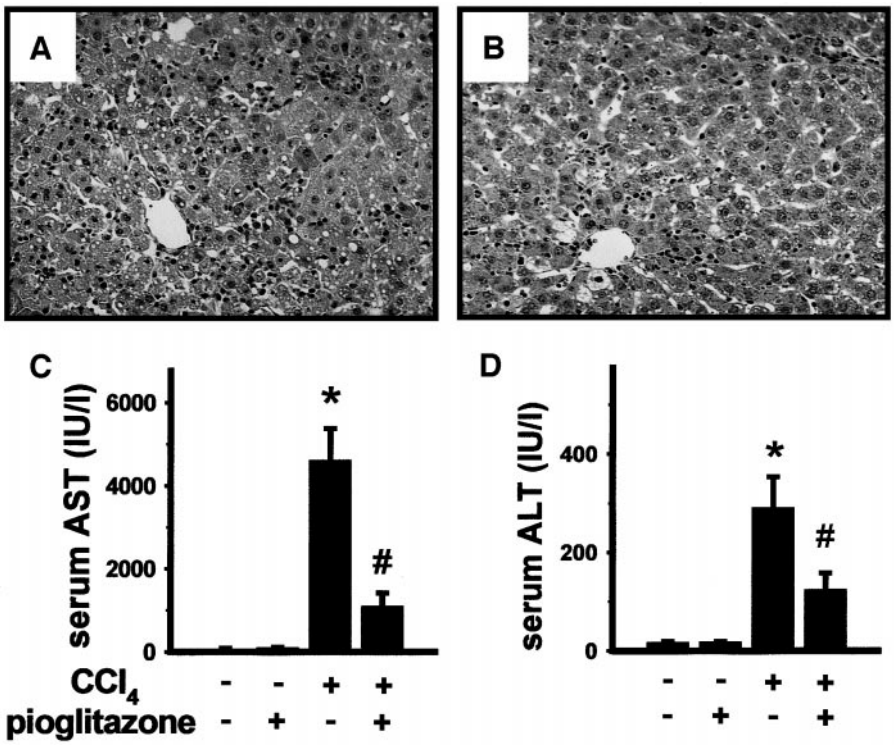

FIG. 3. Effect of pioglitazone on necroinflammatory changes in the liver and serum transaminases levels following acute CCl_4. Experimental design as in Fig. 1 except that liver tissues and serum samples were collected 24 h after treatment with CCl_4 and/or pioglitazone. (A and B) Liver histology. Representative photomicrographs of liver histology from CCl_4 alone (C) and CCl_4 plus pioglitazone (D) are shown (H-E staining, original magnification: ×200). (C and D) Serum transaminases levels. Mean serum AST (C) and ALT values (D) ±SEM in each group are plotted ($n = 8–10$, $*P < 0.05$ vs controls, $\#P < 0.05$ vs CCl_4 alone, by ANOVA on ranks and Dunn's post-hoc test).

caused neither histological changes nor elevations in transaminases.

To further evaluate the changes in inflammatory responses caused by CCl_4, serum TNF-α levels were determined by ELISA (Fig. 4). Coadministration of pioglitazone blunted CCl_4-induced increases in serum TNF-α levels significantly. Taken together, pioglitazone prevented necroinflammatory responses following acute CCl_4 administration.

Effect of Pioglitazone on Transactivation of HSCs in Vitro

To examine the direct effect of pioglitazone on HSC activation, primary cultured HSCs were incubated with various doses of pioglitazone for 3 days (from day 4 to day 7 after inoculation) and the expression of αSMA was detected by indirect immunofluorescence analysis (Fig. 5). The control HSCs at day 7 of culture demonstrated marked expression of αSMA, which appeared to form stress fiber, as expected (Fig. 5A). In contrast, this induction of αSMA *in vitro* was inhibited when cells were incubated with the media containing pioglitazone (1–5 mM) for 3 days in a dose dependent manner (Figs. 5B and 5C). Further, the expression levels of αSMA in these cells were quantitatively analyzed by Western blotting. As shown in Fig. 5D, pioglitazone dose-dependently decreased the expression levels of αSMA in 7-day cultured HSCs.

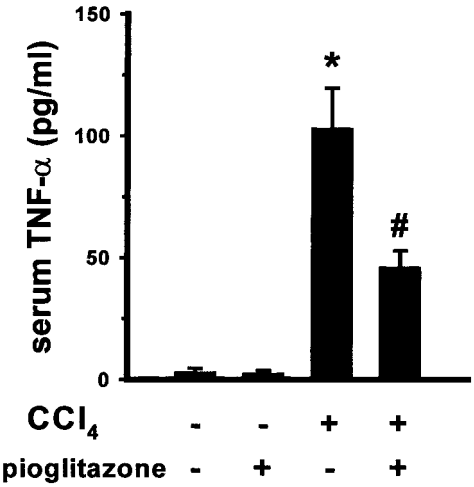

FIG. 4. Effect of pioglitazone on serum TNF-α levels following acute CCl_4. Experimental design as in Fig. 1 except that serum samples were obtained 12 h after CCl_4 and/or pioglitazone. Serum TNF-α levels were determined by ELISA. Mean serum TNF-α levels ± SEM in each group are plotted ($n = 5–6$, $*P < 0.05$ vs controls, $\#P < 0.05$ vs CCl_4 alone, by ANOVA on ranks and Dunn's post-hoc test).

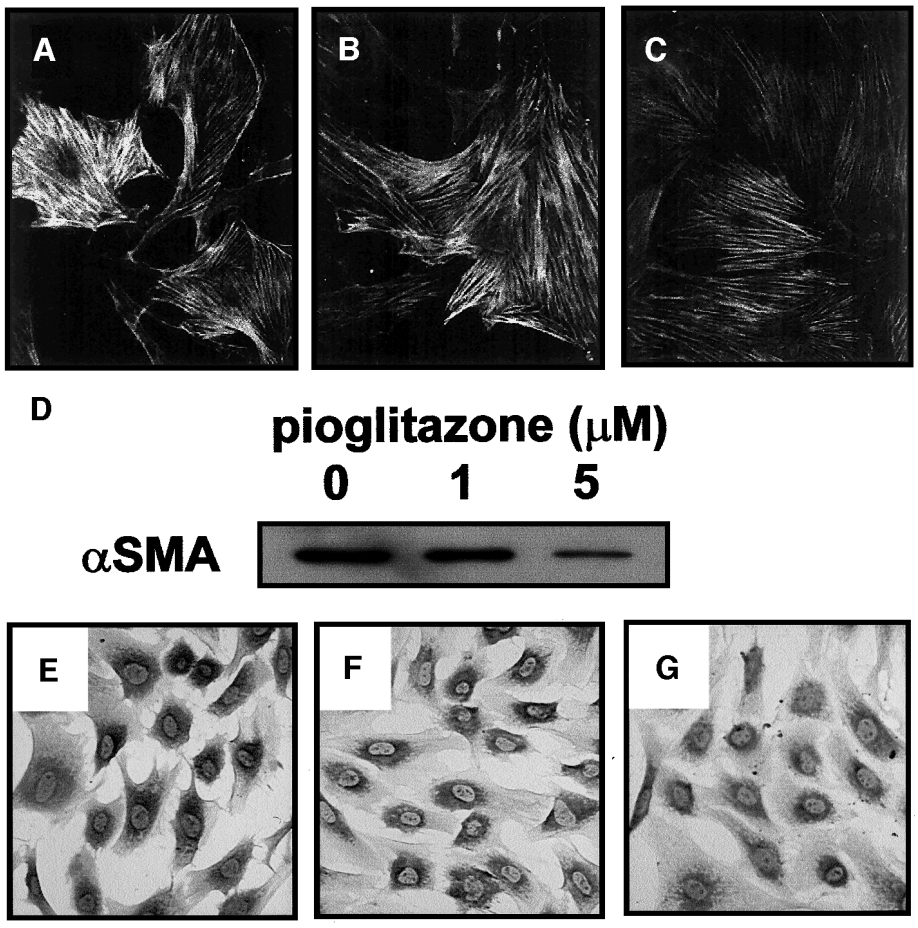

FIG. 5. Effect of pioglitazone on expression of αSMA and type I collagen in isolated hepatic stellate cells (HSCs). (A–C) Expression of αSMA. Primary cultured rat HSCs were incubated with pioglitazone (1–5 μM) for 72 h (from day 4 to day 7 after inoculation), and localization and quantity of αSMA expression was detected by indirect immunofluorescence analysis (A–C) and Western blotting (D) using a monoclonal anti-αSMA antibody, respectively. Representative photographs of immunofluorescence analysis from controls (A), 1 μM (B), and 5 μM (C) of pioglitazone for 72 h (original magnification; ×1000), and the specific, 42-kDa bands for αSMA (D) were shown. (E–G) Expression of type I collagen. Expression of type I collagen in 7-day cultured HSCs was detected by immunocytochemistry using an anti-type I collagen antibody. Representative photomicrographs of controls (E), 1 μM (F), and 5 μM (G) of pioglitazone for 72 h are shown (original magnification; ×400). Similar results were observed in 4 different preparations.

Next, the effect of pioglitazone on expression of type I collagen in transactivated HSCs *in vitro* was evaluated by immunocytochemical staining (Figs. 5E–5G). The control HSCs at day 7 of culture demonstrated marked expression of type I collagen in cytoplasm as expected (Fig. 5E). In contrast, this induction of type I collagen *in vitro* was inhibited by addition of pioglitazone dose-dependently (Figs. 5F and 5G). These observations confirmed that pioglitazone inhibits transactivation and production of type I collagen in primary cultures of HSCs *in vitro*.

DISCUSSION

In the present study, we demonstrated that pioglitazone, a thiazolidinedione derivative, is preventive against necroinflammatory responses and subsequent early-phase profibrotic responses in the liver caused by acute CCl_4 administration. To our knowledge, this is the first report that TZDs prevent hepatic fibrogenesis *in vivo*. The mechanisms of acute CCl_4 hepatotoxicity involve immediate cleavage of CCl_4 by cytochrome P450 2E1 (CYP2E1) in hepatocytes (20), which generates trichloromethyl radical, leading to lipid peroxidation and membrane damage (21). Subsequently, activated hepatic macrophages (Kupffer cells) produce toxic mediators (e.g., inflammatory cytokines, reactive oxygen intermediates, eicosanoids), resulting in the injury of parenchymal cells (22). On the other hand, lines of evidence indicated that PPARγ is implicated in differentiation and inflammatory responses in monocytes/macrophages (16–19). There is controversy, however, in respect to the role of PPARγ in LPS-induced cytokine production in macrophages; it has been re-

ported that TZDs do not inhibit LPS-induced production of TNF-α in macrophages (18, 23). In this study, we evaluated the effect of pioglitazone on necroinflammatory responses following acute CCl_4 administration. Pioglitazone prevented increases in serum transaminases and TNF-α levels following a single CCl_4 injection significantly (Figs. 3 and 4). These observations are in line with the recent report that pioglitazone prevents LPS-induced production of TNF-α and nitric oxide in isolated Kupffer cells (24). It is likely therefore that pioglitazone prevents activation of Kupffer cells during CCl_4 intoxication, thereby decreasing subsequent profibrogenic responses in the liver.

On the other hand, PPARγ is profoundly involved in proliferation and activation of HSCs *in vitro* (9–11). In HSCs, expression levels and activity of PPARγ tend to decrease during activation process, and PPARγ ligands prevent this down-regulation (9, 10). Therefore, it is possible that natural ligands for PPARγ and RXR play important roles in maintaining quiescent phenotype of HSCs, and TZDs exert the similar effect. Further, platelet-derived growth factor (PDGF), the most potent mitogen of HSCs, is believed to promote profibrogenic responses during the healing response of hepatic wound, whereas $15dPGJ_2$ and other TZDs has been demonstrated to prevent PDGF-induced proliferation and chemotaxis of HSCs *in vitro* (10, 11). The mechanism underlying this reaction has not been elucidated completely, while it seems to be independent on downstream signaling pathways of PDGF receptor including Ras/ERK, phosphatidylinositol 3-kinase and expression of proto-oncogenes c-*fos* and c-*myc* (11). It is postulated, therefore, that pioglitazone prevented PDGF-induced proliferation and chemotaxis of HSCs during acute CCl_4 intoxication. Moreover, we confirmed that pioglitazone prevents not only the induction of αSMA, but also the expression of type I collagen in 7-day cultured HSCs (Fig. 5), which is consistent with the previous report that $15dPGJ_2$ and rosiglitazone inhibit promoter activity of α1(I)procollagen gene in isolated HSCs (9). Collectively, these findings support the hypothesis that pioglitazone directly prevents, in part, the transactivation of HSCs *in vivo,* thereby ameliorating the early fibrogenesis in the liver caused by CCl_4.

In conclusion, pioglitazone, a PPARγ ligand, prevents hepatic profibrogenic responses *in vivo* induced by acute CCl_4 administration. It is postulated therefore that PPARγ ligands, including TZDs, are potential therapeutic reagents for prevention and/or treatment of hepatic fibrosis.

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