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Pioglitazone prevents hepatic steatosis, fibrosis, and enzyme-altered lesions in rat liver cirrhosis induced by a choline-deficient L-amino acid-defined diet☆

Kotaro Kawaguchi, Isao Sakaida,* Masako Tsuchiya, Kaoru Omori, Taro Takami, and Kiwamu Okita

Department of Gastroenterology and Hepatology, School of Medicine, Yamaguchi University, Minami-Kogushi 1-1-1 Ube, Yamaguchi 755-8505, Japan

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Abstract

Non-alcoholic steatohepatitis (NASH) may progress to liver cirrhosis, and NASH patients with liver cirrhosis have a risk of development of hepatocellular carcinoma. Peroxisome proliferator-activated receptor (PPAR) γ ligand has recently been reported to have improved the condition of patients with NASH. The aim of this study was to investigate whether pioglitazone, a PPARγ ligand, has any influence on the animal model of NASH as well as isolated hepatic stellate cells. In vivo, the effects of pioglitazone were examined using the choline-deficient L-amino acid-defined (CDAA)-diet liver fibrosis model. After two weeks, pioglitazone improved hepatic steatosis, prevented liver fibrosis, and reduced preneoplastic lesions in the liver after 10 weeks. Pioglitazone reduced the expression of TIMP-1 and TIMP-2 mRNA without changing MMP-13 mRNA expression compared to the liver fed a CDAA diet alone. In vitro, pioglitazone prevented the activation of hepatic stellate cells resulting in reducing the expression of type I procollagen, MMP-2, TIMP-1, and TIMP-2 mRNA with increased MMP-13 mRNA expression. These results indicate that pioglitazone may be one of the candidates for the benefit drugs for the liver disease of patients with NASH.

Keywords: Peroxisome proliferator-activated receptor-γ; Non-alcoholic steatohepatitis; Hepatic stellate cell; Fibrosis; Preneoplastic lesion

In 1980, Ludwig et al. [2–4] coined the term non-alcoholic steatohepatitis (NASH) as a form of liver disease that is histologically indistinguishable from alcoholic hepatitis but occurs in patients without history of alcohol abuse [1]. Some patients with NASH progress liver cirrhosis. And it has been reported NASH patient with liver cirrhosis may develop hepatocellular carcinoma [5]. However, the pathogenesis of NASH is poorly understood, insulin resistance may be strongly associated with NASH because most patients with NASH are obese and have Type II diabetes mellitus [6].

Pioglitazone, one member of thiazolidinedione, is used to improve insulin resistance in Type II diabetes mellitus as the ligand of the peroxisomal proliferator-activated receptor (PPAR) γ [7]. PPARγ ligand has recently been reported improving hepatic steatosis, alanine aminotransferase elevation, and insulin sensitivity in patient with NASH [8,9]. Then it has been reported preventing hepatic fibrogenesis in some animal models of liver fibrosis by inhibiting activation of hepatic stellate cells [10–13]. But the effect of PPARγ ligand on the expressions of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) in fibrotic liver leading to carcinogenesis [14–16] has not been assessed yet.

In this study, we used choline-deficient L-amino acid-defined (CDAA) diet-induced liver fibrosis model, one of the animal models of NASH [17]. This study demonstrated that pioglitazone prevented hepatic steatosis,

☆ *Abbreviations:* αSMA, α smooth muscle actin; CDAA, choline-deficient L-amino acid-defined; CSAA, choline-supplemented L-amino acid-defined; NASH, nonalcoholic steatohepatitis; HSC, hepatic stellate cell; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; PPAR, peroxisome proliferator-activated receptor.

* Corresponding author. Fax: +81-836-22-2240.
E-mail address: sakaida@yamaguchi-u.ac.jp (I. Sakaida).

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fibrosis with reduced expression of TIMP-1 and TIMP-2 resulting in the prevention of development of enzyme-altered lesions. Also we demonstrated the direct preventive effect of pioglitazone on the activation of hepatic stellate cells leading to the prevention of reduction of MMP-13 mRNA expression.

Materials and methods

In vivo

Animals. Male Wistar rats, 6 weeks of age and weighing 140–150 g (Nippon SLC, Shizuoka, Japan), were obtained, quarantined for 1 week, and housed in a room under controlled temperature (25 °C), humidity, and lighting (12 h light, 12 h dark). Access to food and tap water was ad libitum throughout the study period.

Drug and diets. Pioglitazone was purchased from Takeda Pharmaceutical Company (Osaka, Japan). The CDAA diets were obtained in powdered form (Dyets, Bethlehem, PA, USA; product Nos. 518753 and 518754). The detailed compositions of these diets have been described in previous reports [18]. Pioglitazone in powder was mixed uniformly into the CDAA diet at concentrations of 0–0.01% (w/w). Normal diet groups were fed a choline-supplemented L-amino acid-defined (CSAA) diet with or without 0.01% (w/w) pioglitazone.

Experimental protocol. The total study periods were 2 and 10 weeks. With 2-week experiment, the two groups of six rats each received a CSAA diet with or without 0.01% (w/w) pioglitazone, and other two groups of 12 rats each received a CDAA diet with or without 0.01% (w/w) pioglitazone. In 10-week experiment, the two groups of six rats each received a CSAA diet with or without 0.01% (w/w) pioglitazone, and three groups of 12 rats each received a CDAA diet containing 0%, 0.001%, and 0.01% (w/w) pioglitazone. To equalize the total food intake in all groups, additional food was not supplied until all food in all groups had been consumed. Food intake of each group was measured.

Measurement of the content of triacylglycerol in the liver tissue. With 2-week experiment, the content of triacylglycerol in the liver tissue was measured in all groups. The triacylglycerol in the liver tissue was extracted by the method of Folch et al. [19].

Serum marker measurement. In all experiments, serum ALT, alkaline phosphatase (ALP), Triacylglycerol (TG), hyaluronic acid, and bile acid were measured.

Histology and immunohistochemical examination. Sections 5-μm thick of the right lobe of all rat liver, fixed in 10% formalin for 24 h and embedded in paraffin, were processed for sirius red staining. α-Smooth muscle actin (αSMA) for the detection of activated stellate cells, and glutathione *S*-transferase placental form (GST-P)-positive lesions (as preneoplastic lesions) were immunohistochemically assessed by the avidin-biotin-peroxidase complex method, as described previously [15]. αSMA and GSTP-positive cells in the liver were quantified using a Provis microscope (Olympus, Tokyo, Japan) equipped with a charge coupled devise (CCD) camera, and subjected to computer-assisted image analysis with MetaMorph software (Universal Imaging Corporation, Downingtown, PA). Ten randomly selected different areas per one specimen were analyzed. The area of sirius red positive area and αSMA-positive cells was expressed as the percentage of the total area of the specimen. The size and numbers of GST-P-positive lesions were counted in each specimen.

Real-time quantitative PCR. Expression of type I procollagen, MMP-2, MMP-13, TIMP-1, and TIMP-2 mRNA was evaluated by real-time PCR as described previously [20]. Briefly, total RNA from rat liver was isolated with an RNeasy-kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. For cDNA synthesis, Taqman reverse transcription reagents were used as described in the manufacturer's manual (Roche Diagnostics, Indianapolis, IN, USA). Relative quantification of gene expression was performed as described in the manual using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The threshold cycle and the standard curve method were used for calculating the relative amount of the target RNA. Light Cycler Q-PCR (Roche Diagnostics) was performed (operating system version 3.0) in 13 μl mixtures containing 2 μl of faststart DNA Sybr greenI, 25 mM $MgCl_2$, 10 μM each primer, and 5 μl of extracted DNA. The reaction was performed with preliminary denaturation for 10 min at 95 °C (slope 20 °C/s), followed by 40 cycles of denaturation at 95 °C for 15 s (slope 20 °C /s), annealing at 68 °C for 5 s (slope, 20 °C/s), primer extension at 72 °C for 10 s (slope 20 °C/s), and product detection at 72 °C for 60 s (slope 20 °C/s). The synthesis kit for RT-PCR (AMV) and Light Cycler-Fast Start DNA Master SYBR Green I was purchased from Roche Diagnostics. Advantage PCR polymerase was obtained from Clonteck Laboratories (Palo Alto, CA).

Plasmids for real-time PCR. Type I procollagen: sense (5′-agcggtga agaaggaaagagagg-3′) and antisense (5′-caataggaccagaaggaccagca-3′), MMP-2: sense (5′-gccctcccctgatgctgata-3′),and antisense (5′-gtcactgtcc gccaaataaacc-3′), MMP-13: sense (5′-tggtcttctggcacacgcttt-3′) and antisense (5′-gcttagggttggggtcttcatc-3′), TIMP-1: sense (5′-ccccaacccac ccacagacagc-3′) and antisense (5′-cgctgcggttctgggacttgtg-3′), TIMP-2: sense (5′-cagggccaaagcagtgagcgagaa-3′) and antisense (5′-tcttgccatctcc ttccgccttcc-3′), and GAPDH: sense (5′-ggcaagttcaacggcaca gtc-3′) and antisense (5′-agcaccagcatcaccccattt-3′) were used. The inserts were confirmed by DNA sequencing and used as probes for real-time PCR analysis.

In vitro

Isolation of hepatic stellate cells. Rat hepatic stellate cells were isolated as described previously [21,22] with some modifications. In brief, the liver was perfused in situ via the portal vein with Ca^{2+}, Mg^{2+}-free Kreb–Ringer (KR) solution followed by 0.1% pronase E (Merck, Darmstadt, Germany) and then 0.032% collagenase (Wako Pure Chemical, Osaka, Japan) solution at 37 °C. The digested liver was excited and minced, and incubated in KR solution containing 0.08% pronase E, 0.04% collagenase, and 20 μg/ml DNase (Boehringer–Mannheim, Mannheim, Germany) for 30 min at 37 °C (pH 7.3). The resulting suspension was then passed through a nylon mesh. The filtrate was centrifuged at 450*g* for 8 min. The fraction enriched with hepatic stellate cells was finally obtained by centrifugation in 8.2% Nycodenz (Nycomed Pharma AS, Oslo, Norway) solution at 1400*g* for 20 min at 4 °C. Hepatic stellate cells in the upper white layer were washed by centrifugation at 450*g* for 8 min, suspended in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharm., Tokyo, Japan) containing 10% fetal calf serum (FCS) (Commonwealth Serum Laboratories, Melbourne, Australia), and supplemented with 100 U/ml penicillin, 100 mU/ml streptomycin (Gibco Laboratories, Life Technologies, Grand Island, NY, USA).

Yields were $1.0–1.5 \times 10^7$ cells/rat. Cell viability was always over 95% as determined by the trypan blue exclusion test. Cell purity was more than 95% as assessed by the presence of yellow-colored droplets and desmin immunoreactivity after overnight incubation.

Preparation of culture medium with pioglitazone. Pioglitazone (Takeda Chemical Industries, Osaka, Japan) was dissolved in DMSO to the concentration of 50 mM and then passed through 0.22 μm filters for sterilization. This solution was diluted with DMEM to the final concentration of 20 μM. The pH values and osmotic pressures of culture media with or without pioglitazone were adjusted within physiological ranges.

Final concentration of DMSO in the medium was 0.1% (v/v).

Culture of hepatic stellate cells. Freshly isolated hepatic stellate cells suspended in culture medium were seeded at a density of 5.0×10^5 cells/ml in monolayer culture on uncoated 60-mm plastic dishes (Iwaki Glass, Tokyo, Japan). All cultures were incubated at 37 °C in a

humidified atmosphere of 5% CO_2 and 95% air. After incubation for 4 h, non-adherent cells were removed with a pipette and the culture medium was replaced with medium containing pioglitazone or the same concentration of DMSO as a control. The medium with or without pioglitazone was changed every 24 h and cell culture was continued up to 4 days.

Western blot analysis. The expression of αSMA protein was determined by Western blot analysis. Anti-αSMA monoclonal antibody (Dako Japan, Kyoto, Japan) was employed as primary antibody. Secondary antibodies for αSMA and ECL Western blotting detection reagents were from Amersham–Pharmacia Biotech (Piscataway, NJ, USA). Briefly, the treated HSCs were suspended at 4 °C in 0.5 ml of a lysis buffer consisting of 20 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The cell lysates containing 80 μg of total protein were analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting. Proteins were electrophoretically transferred to a polyvinylidine difluoride membrane (Bio-Rad, Hercules, CA) and probed with anti-αSMA. The bands were visualized with an ECL Western Blotting Detection System (Amersham) for the development of immunoblots using a horseradish peroxidase-conjugated secondary antibody (Amersham). All experiments were repeated at least three times with different batches of the cell samples and results were fully reproducible.

Real-time quantitative PCR. Briefly, total RNA from treated HSCs was isolated with an RNeasy-kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Expression of type I procollagen, MMP-2, MMP-13, TIMP-1, and TIMP-2 mRNA was evaluated by real-time PCR as in vivo experimental method.

Statistical methods. Results are expressed as means ± SD, and the data obtained were evaluated by analysis of variance as appropriate. The level of significance was set at 5%.

Results

In vivo

Effect of pioglitazone with 2 weeks experiment

In this model, the livers of rats fed the CDAA diet for 2 weeks developed severe steatosis and showed an increased liver weight of 15.6 ± 1.3 g, compared with 8.5 ± 0.3 g for rats fed a CSAA diet (Table 1). Concurrent administration of 0.01% (w/w) pioglitazone for rats fed the CDAA diet significantly reduced this increase in liver weight to 12.6 ± 1.4 g (Table 1). The size of fat drops in hepatocyte fed the CDAA diet with 0.01% (w/w) pioglitazone seemed to be smaller than that of a rat fed the CDAA diet without pioglitazone (Fig. 1). Actually rats fed the CDAA diet with 0.01% (w/w) pioglitazone showed the decreased content of triacylglycerol in the liver tissue of 337.4 ± 13.5 mg/g wet weight, compared with 369.4 ± 23.9 mg/g wet weight for rats fed CDAA diet without pioglitazone (Table 1). This finding indicated that pioglitazone improved hepatic steatosis in the early stage of this model.

Table 1
Effect of pioglitazone on characteristics of rats (2 weeks)[a]

Treatment (no. of rats)	Body weight (g)	Liver weight (g)	TG in the liver tissue (mg/g wet weight)
CSAA (6)	216 ± 8	8.5 ± 0.3	2.5 ± 1.0
CDAA (6)	203 ± 7[b]	15.6 ± 1.3[c]	369.4 ± 24.0[c]
CDAA + pio (6)	206 ± 6[b]	12.6 ± 1.4[c,e]	337.4 ± 13.5[c,d]

Note. Values are means ± SD. Control, CSAA diet; CDAA, CDAA diet without pioglitazone; and CDAA + pio, CDAA diet with 0.01% pioglitazone .

[a] Rats were fed the normal diet or the CDAA diet with 0% and 0.01% pioglitazone. After 2 weeks, rats were sacrificed and body weight, liver weight, and triglyceride in the liver were measured as described in the text.

[b] $p < 0.05$ vs. normal diet.

[c] $p < 0.01$ vs. normal diet.

[d] $p < 0.05$ vs. CDAA diet without pioglitazone.

[e] $p < 0.01$ vs. CDAA diet without pioglitazone.

Effect of pioglitazone with 10 weeks experiment

Serum markers of liver fibrosis. In this model, rats fed the CDAA diet for 10 weeks showed an increased serum hyaluronic acid level of 137 ± 44 ng/ml, compared with 60 ± 22 ng/ml for rats fed the CSAA diet (Table 2).

Also pioglitazone reduced serum ALP and bile acid levels in a dose dependent manner compared with those of rats fed the CDAA diet without pioglitazone (Table 2).

Concurrent administration of 0.001 and 0.01% (w/w) pioglitazone significantly reduced this increased hyaluronic acid in a dose dependent manner (Table 2). In addition, 0.001% and 0.01% (w/w) pioglitazone did not reduce the increase in serum ALT level (Table 2). The short-time (2 weeks) experiment also indicated that addition of 0.01% pioglitazone to the CDAA diet did not reduce the serum ALT level (1194 ± 392 U/L) compared with the CDAA diet without pioglitazone (1058 ± 452 U/L). Thus, the inhibition of fibrosis by pioglitazone could not be attributed to the prevention of hepatocyte cell injury. The administration of 0.01% pioglitazone for 10 weeks to rats fed CSAA diet did not affect the serum ALT level compared with the CSAA diet without pioglitazone (CSAA diet: 49 ± 12 U/L, CSAA diet with pioglitazone: 36 ± 5 U/L). These findings indicated that 0.01% (w/w) pioglitazone did not exhibit any hepatotoxicity.

Histological findings. In this model, the livers of rats fed the CDAA diet for 10 weeks showed extensive accumulation of extracellular matrix. Concurrent administration of 0.001% and 0.01% (w/w) pioglitazone prevented this accumulation of extracellular matrix in a dose dependent manner (Fig. 2). Activated stellate cells, which express αSMA and therefore also called myofibroblast-like cells, showed the prominent proliferation in the livers of rats fed CDAA diet for 10 weeks. These cells were not seen in the livers of rats fed a CSAA diet (data not shown). The addition of 0.001% and 0.01% pioglitazone also reduced the area of αSMA-positive cells in the liver of rats fed a CDAA diet in a dose dependent manner (Fig. 3). Quantitative analysis showed that 0.001% and 0.01% pioglitazone significantly

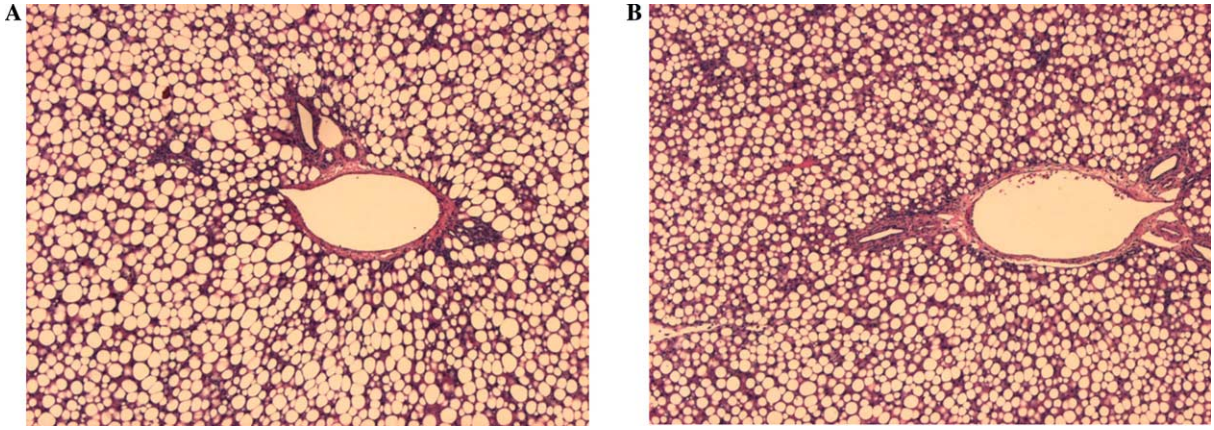

Fig. 1. Photomicrographs of liver sections stained with HE from a male Wistar rat fed the CDAA diet for 2 weeks (A); and from a rat fed the CDAA diet with 0.01% pioglitazone for 2 weeks (B). Magnification: 40×.

Table 2
Effect of pioglitazone on serum markers[a]

Treatment (no. of rats)	Hyaluronic acid (ng/ml)	Total bile acid (μmol/L)	ALP (U/L)	ALT (U/L)
CSAA (6)	60 ± 22	4 ± 2	398 ± 25	43 ± 6
CDAA (12)	137 ± 44[b]	55 ± 19[c]	780 ± 122[c]	512 ± 87[c]
CDAA + pio-low (12)	85 ± 26[d]	44 ± 11[c]	618 ± 45[c,d]	625 ± 180[c]
CDAA + pio-high (12)	58 ± 12[e,f]	30 ± 7[c,d,f]	582 ± 31[c,d]	578 ± 148[c]

Note. Values are means ± SD. Control, CSAA diet; CDAA, CDAA diet without pioglitazone; CDAA + pio-low, CDAA diet with 0.001% pioglitazone; and CDAA + pio-high, CDAA diet with 0.01% pioglitazone.

[a] Rats were fed the normal diet or the CDAA diet with 0%, 0.001%, and 0.01% pioglitazone. After 10 weeks, rats were sacrificed and serum markers were measured as described in the text.

[b] $p < 0.05$ vs. normal diet.

[c] $p < 0.01$ vs. normal diet.

[d] $p < 0.05$ vs. CDAA diet without pioglitazone.

[e] $p < 0.01$ vs. CDAA diet without pioglitazone.

[f] $p < 0.05$ vs. CDAA diet with 0.001% pioglitazone.

reduced the percent area of sirius red and αSMA-positive cells compared with the CDAA diet without pioglitazone (Table 3).

These findings suggested that the prevention of fibrogenesis by pioglitazone was related to its inhibitory effect on the activation of hepatic stellate cells.

Effect of pioglitazone on GST-P-positive lesions

In this model at 10 weeks, GST-P-positive lesions consisted mainly of these nodules. The results of quantitative analysis of GST-P-positive lesions in the liver at the end of the study are shown in Table 4. Administration of the CDAA diet for 10 weeks was associated with the development of a large number of GST-P-positive lesions. The concomitant administration of 0.01% pioglitazone significantly reduced the number and the area of GST-P-positive lesions, compared with the livers of rats fed CDAA diet without pioglitazone (Table 4) (Fig. 4).

Effect of pioglitazone on fibrogenesis in rat liver

To investigate the effect of pioglitazone on fibrogenesis and fibrolysis in rat liver, we assessed mRNA expressions of type I procollagen, MMP-2, MMP-13, TIMP-1, and TIMP-2 by real-time PCR. The administration of 0.01% pioglitazone to rats fed a CDAA diet significantly reduced the expression of type I procollagen, αSMA?, MMP-2, TIMP-1, and TIMP-2 mRNA in the liver, compared with those of rats fed a CDAA diet without pioglitazone (Fig. 5).

On the other hand, the expression of MMP-13 mRNA in the liver did not show significant difference between both groups (Fig. 5). These results indicate that pioglitazone reduced the expression of type I procollagen, TIMP-1, and TIMP-2 mRNA in the liver without affecting the expression of MMP-13 mRNA, which mainly hydrolyzes extracellular matrix. Thus, these results indicate the prevention of activation of stellate cell by pioglitazone.

In vitro

Effect of pioglitazone on the expression of αSMA protein in HSCs

To confirm the inhibitory effect on the activation of HSCs with pioglitazone, we measured the expression of

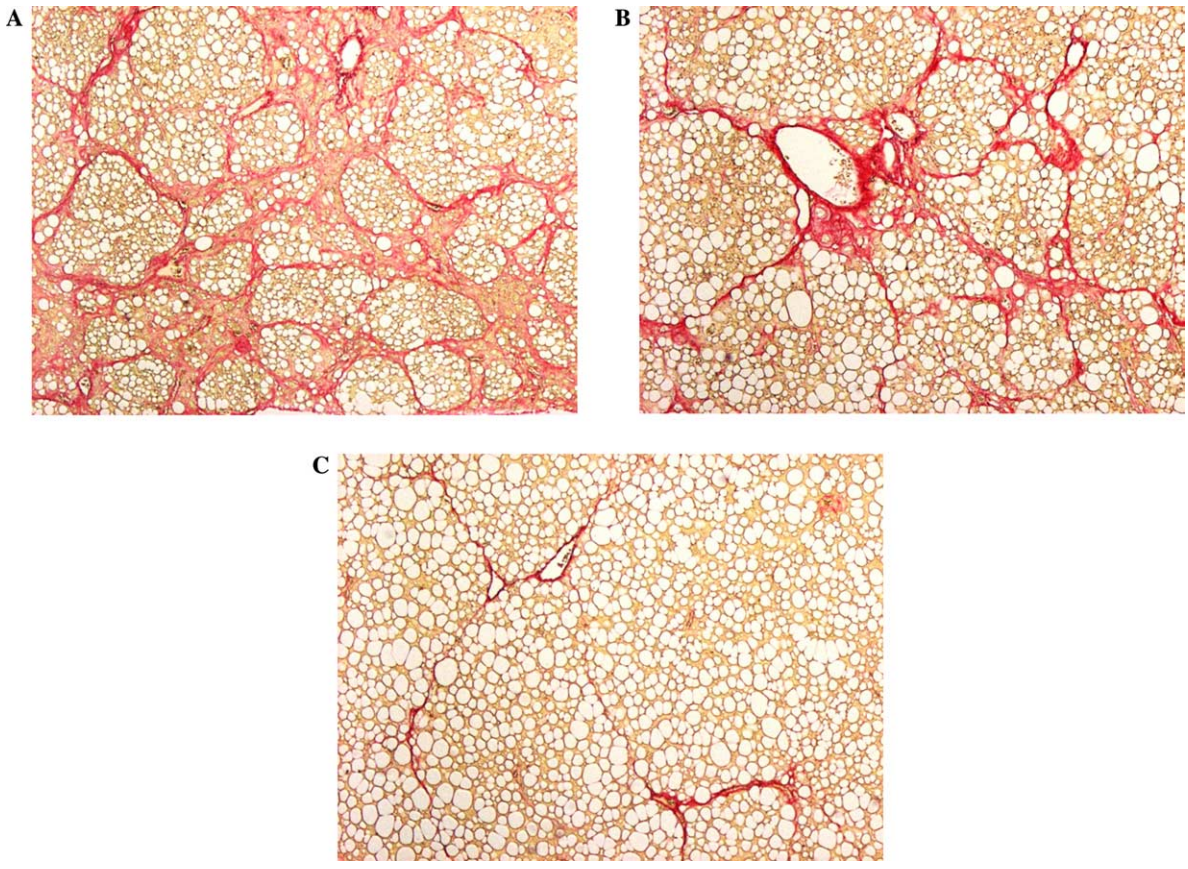

Fig. 2. Photomicrographs of liver sections stained with sirius red from a male Wistar rat fed the CDAA diet for 10 weeks (A); and from a rat fed the CDAA diet with 0.001% and 0.01% pioglitazone for 10 weeks (B, C). Magnification: 40×.

αSMA protein in HSCs by Western blot. HSCs treated with pioglitazone reduced the expression of αSMA protein, compared with control group in a dose dependent manner (Fig. 6).

Effect of pioglitazone on fibrogenesis and fibrolysis in HSCs

As 20 μM of pioglitazone showed strong inhibitory expression of αSMA expression of isolated stellate cell, we used this dose to investigate the effect of pioglitazone on fibrogenesis and fibrolysis in HSCs. We measured the mRNA expressions of type I procollagen, αSMA, MMP-2, MMP-13, TIMP-1, and TIMP-2 by real-time PCR. In time course, the expressions of type I procollagen, MMP-2, TIMP-1, and TIMP-2 were increased with activation of HSCs. Treatment of 20 μM pioglitazone significantly reduced these increased mRNA expressions, compared with control group (Fig. 7). On the other hand, the expression of MMP-13 mRNA in HSCs rapidly decreased within 4 days after isolation. Treatment of 20 μM pioglitazone significantly prevented this decrease of MMMP-13 mRNA expression, compared with control group (Fig. 7). These results indicate that pioglitazone prevented hepatic fibrogenesis by inhibiting activation of HSCs obviously. In addition, pioglitazone reduced the increase of the expression of TIMP-1 and TIMP-2 mRNA with keeping the expression of MMP-13 mRNA.

Discussion

Fatty liver occurs in several days when rats are fed with CDDA diet, followed by the progression to liver cirrhosis associated with marked fibrosis in several weeks and then ultimately to hepatocellular carcinoma [16]. Based on this observation, we selected rats with hepatic fibrosis due to CDDA diet as an animal model of NASH in the present study.

In this model, pioglitazone improved fatty liver after 2 weeks. The two-hit theory has been proposed to explain the mechanism for NASH [23], and pioglitazone has been suggested to improve NASH by inhibiting fatty liver (first hit). PPARγ-ligands have been reported to improve fatty liver, but there has been no clear explanation about the mechanism [8,9,24]. In a study designed to elucidate this mechanism, pioglitazone was found to serve as a ligand for PPARα, as well as PPARγ [25], and it was suggested that pioglitazone accelerates β-oxidation and improves fatty liver due to its effect as a

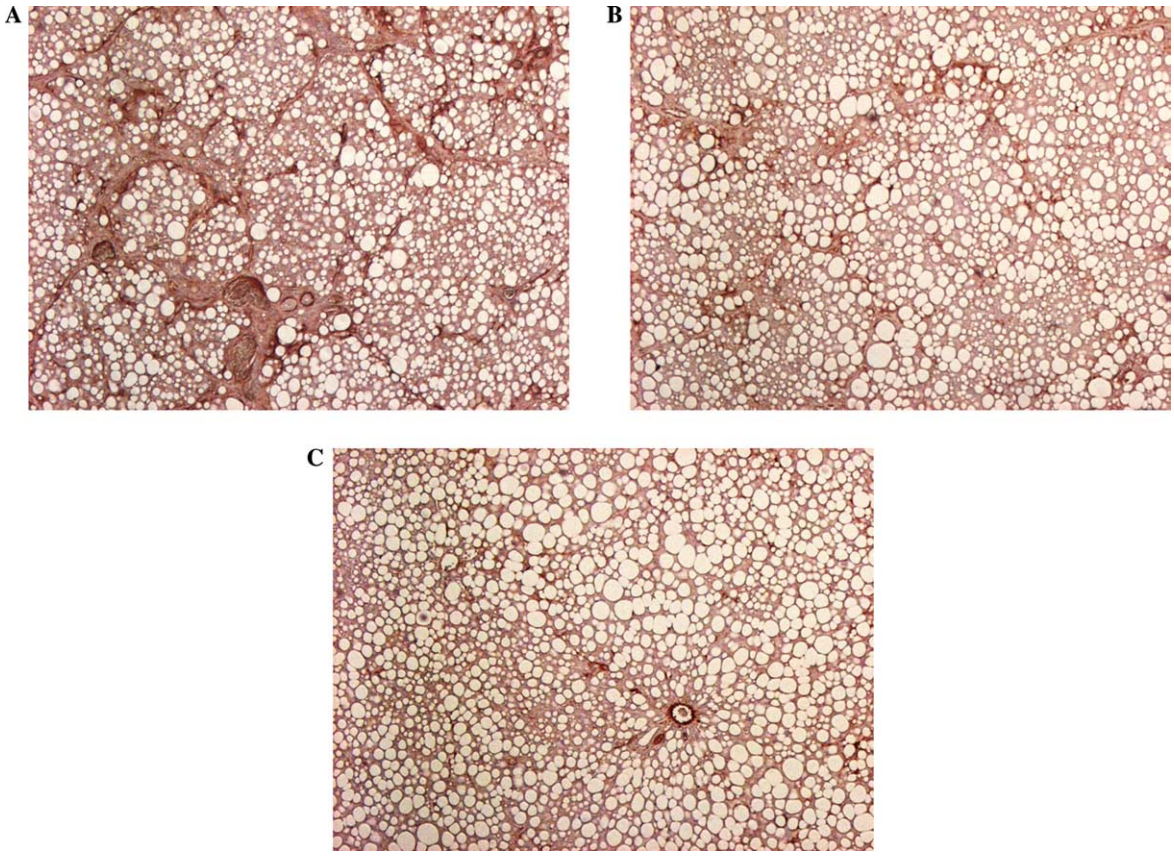

Fig. 3. Photomicrographs of liver sections stained with anti-αSMA muscle actin antibody from a male Wistar rat fed the CDAA diet for 10 weeks (A); and from a rat fed the CDAA diet with 0.001% and 0.01% pioglitazone for 10 weeks (B, C). Magnification: 40×.

Table 3
Effect of pioglitazone on α-smooth muscle actin-positive and sirius red-positive area[a]

Treatment (no. of rats)	α-SMA-positive area (%)	Sirius red-positive area (%)
CDAA (12)	5.41 ± 1.05	9.5 ± 1.2
CDAA + pio-low (12)	3.06 ± 1.04[b]	7.4 ± 0.7[b]
CDAA + pio-high (12)	1.08 ± 0.45[b,c]	4.7 ± 0.6[b,c]

Note. Values are means ± SD. CDAA, CDAA diet without pioglitazone; CDAA + pio-low, CDAA diet with 0.001% pioglitazone; and CDAA + pio-high, CDAA diet with 0.01% pioglitazone.

[a] Rats were fed the CDAA diet with 0%, 0.001%, and 0.01% pioglitazone. After 10 weeks, rats were sacrificed. α-Smooth muscle actin-positive and Sirius red positive area were measured as described in the text.

[b] $p < 0.01$ vs. CDAA diet without pioglitazone.

[c] $p < 0.01$ vs. CDAA diet with 0.001% pioglitazone.

Table 4
Effect of pioglitazone on GST-P-positive lesions[a]

Treatment (no. of rats)	GST-P-positive lesions	
	Area (μm²/section)	Number (/section)
CDAA (12)	201,633 ± 61,950	5.7 ± 2.4
CDAA + pio-high (12)	108,835 ± 77,201[b]	2.7 ± 1.6[b]

Note. Values are means ± SD. CDAA, CDAA diet without pioglitazone; CDAA + pio-high, CDAA diet with 0.01% pioglitazone.

[a] Rats were fed the CDAA diet with 0%, 0.001%, and 0.01% pioglitazone. After 10 weeks, rats were sacrificed. GST-P-positive area and numbers in each sections were measured as described in the text.

[b] $p < 0.05$ vs. CDAA diet without pioglitazone.

ligand for PPRAα. However, further studies seem to be necessary to determine how pioglitazone improves fatty liver. Because studies conducted to date have shown that NASH is likely to progress to liver cirrhosis [2–4], it is important to well control hepatic fibrosis in treating patients with NASH. In the present study, pioglitazone concentration-dependently reduced sirius red-positive lesions in 10 weeks. Pioglitazone was thought to inhibit hepatic fibrosis by suppressing activation of hepatic stellate cells since immunohistochemistry showed similar reductions in αSMA-positive cells and real-time quantitative PCR showed reductions in type I procollagen mRNA. Pioglitazone was reported to inhibit hepatic fibrosis by inhibiting the expression of type I procollagen in various experimental animal models for hepatic disorders, but there has been no report to date on the balance of hepatic MMPs and TIMPs, which are believed to play a crucial role in the formation of hepatic fibrosis. In the present study, we demonstrated, by real-time quantitative PCR, that pioglitazone markedly inhibits TIMP-1 and TIMP-2 mRNA in 10 weeks in this

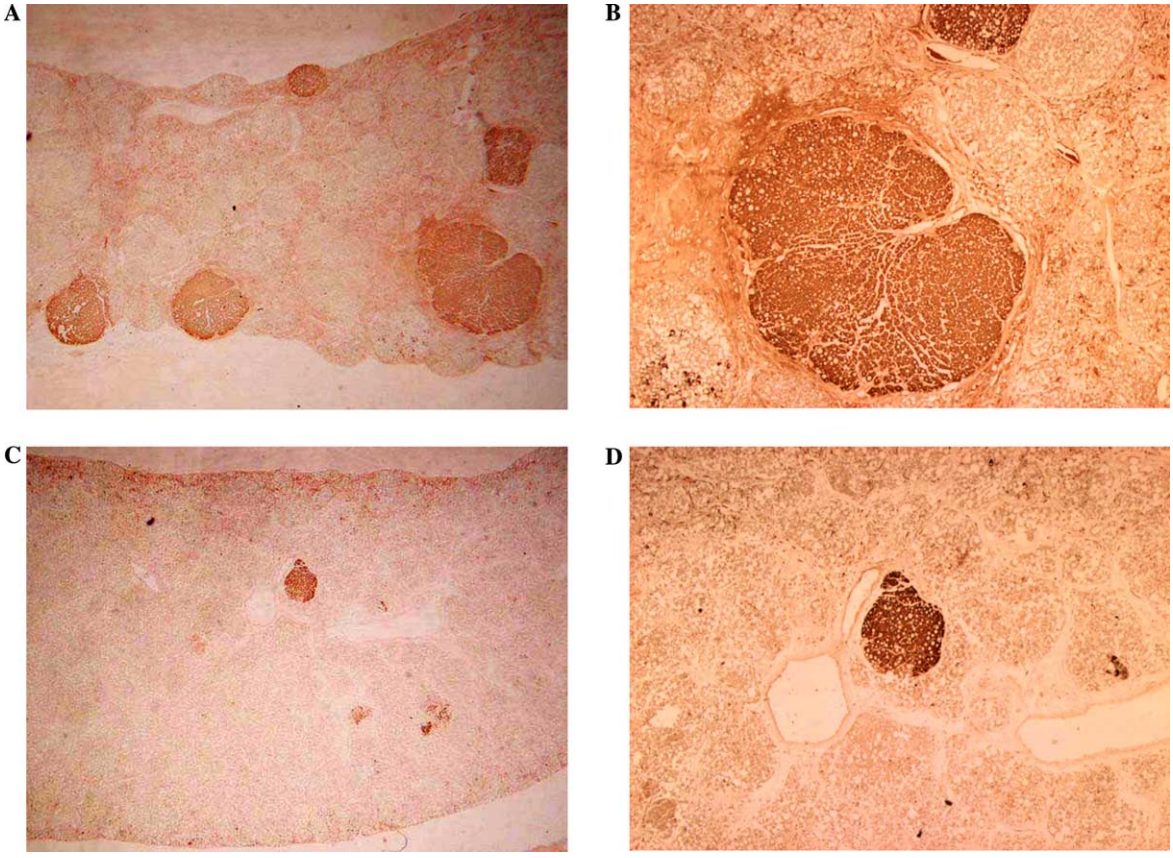

Fig. 4. Photomicrographs of GST-P-positive nodules in a liver section from a male Wistar rat fed the CDAA diet for 10 weeks (A, B); and from a rat fed the CDAA diet with 0.01% pioglitazone for 10 weeks (C and D). Magnification; A, C, 4×; B, D, 40×.

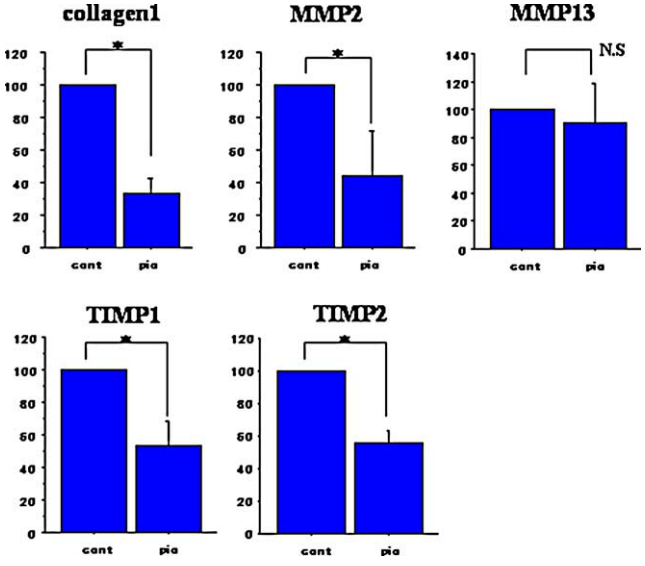

Fig. 5. mRNA expression of type I procollagen, MMPs and TIMPs in the liver. The values are means ± SD of 12 rats each. Cont: rat fed CDAA diet without pioglitazone for 10 weeks Pio: rat fed CDAA diet with 0.01% pioglitazone for 10 weeks $*P < 0.01$.

model. Pioglitazone also inhibited the expression of MMP-2 mRNA. Because MMP-2 has been reported to increase when hepatic stellate cells are activated [26], the

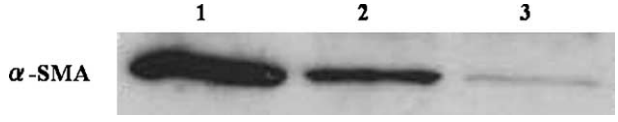

Fig. 6. After isolation, rat primally hepatic stellate cells were incubated with DMEM for 4 h and change medium with various concentrations of pioglitazone (DMSO only, 10 μM, and 20 μM). Then after 24 h, the measurement of expression of αSMA protein was performed by Western blot as described in the text. Lane 1: control (DMSO only); Lane 2: 10 μM pioglitazone; and Lane 3: 20 μM pioglitazone. The figure shows a representative example of three independent experiments.

decreased expression of MMP-2 mRNA was thought to be attributable to the inhibition of stellate cell activation by pioglitazone. No significant difference was noted between the pioglitazone and control groups in the expression of MMP-13 mRNA, which can degrade fibrous collagens, e.g., types I and III collagens, the main players in the formation of hepatic fibrosis. These findings are thought to be consistent with a previous report [26] which indicated that pioglitazone induces fibrolysis in the liver, because it significantly inhibited the expression of TIMPs without causing any marked changes in the expression of MMP-13 leading relatively to the degradation of extracellular matrix.

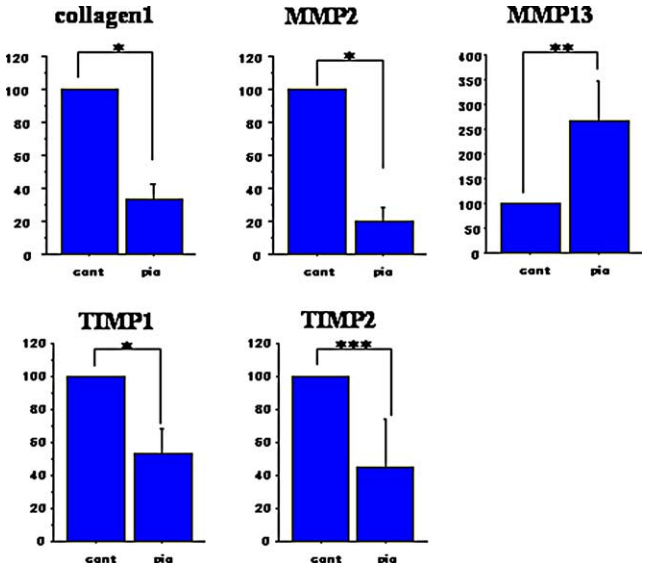

Fig. 7. mRNA expression of type I procollagen, MMPs, and TIMPs in rat primally hepatic stellate cells. The values are means ± SD of three cultures. Cont: HSCs were incubated in DMEM with DMSO only for 4 days. Pio: HSCs were incubated in DMEM with 20 μM pioglitazone for 4 days. *$P < 0.01$; **$P < 0.05$.

We also conducted an in vitro experiment to evaluate the direct effects of pioglitazone on HSCs. Pioglitazone has been reported [12] to inhibit the expression of αSMA and type I procollagen in vitro as well by inhibiting the activation of HSCs, but there has been no report on its effects on the expression of MMPs or TIMPs. When added to isolated HSCs, pioglitazone markedly inhibited the expression of TIMP-1, TIMP-2, and MMP-2 mRNA as in the in vivo experiment. Because their expression increases with the progression of hepatic fibrosis in vivo and the activation of cultured HSCs in vitro [26], this inhibition was thought to be due to inhibition of the activation of HSCs by pioglitazone. All these findings obtained by us suggested that the inhibition of the expression of MMP-2, TIMP-1, and TIMP-2 in vivo was due to inhibition of the activation of HSCs by pioglitazone. The expression of MMP-13 mRNA in isolated HSCs significantly increased in the pioglitazone group. This increase was also thought to be attributable to the inhibition of stellate cell activation by pioglitazone because the expression of MMP-13 has been reported to rapidly decrease in vitro as hepatic stellate cells are activated [26]. Thus, pioglitazone seemed to prevent the loss of MMP-13 mRNA expression. In any case, pioglitazone was thought to be effective in inhibiting hepatic fibrosis in this animal model of NASH.

The presence of hepatocellular carcinoma has been reported in patients with NASH which has progressed to hepatic cirrhosis including our case [5,27,28]. In rats with hepatic fibrosis fed with CDAA diet, GST-P-positive preneoplastic lesions occur in the liver as fatty liver and hepatic fibrosis progress, showing a course similar to that seen with carcinogenesis in NASH patients. In the present study, pioglitazone significantly inhibited both the number and size of GST-P-positive preneoplastic lesions in the 10th week. This finding suggests that pioglitazone inhibits not only hepatic fibrosis but also carcinogenesis in NASH patients. In a previous study, we showed that pre-existing fibrosis with the activated stellate cells accelerates the development of preneoplastic lesions in this model [16]. Thus, inhibition of neoplastic lesions was thought to be due to inhibition of hepatic fibrosis by pioglitazone. On the other hand, a recent study has shown the increases of MMP-2 expression in hepatocellular carcinoma tissues which may suggest that carcinogenesis in the liver is promoted with tissue remodeling and neovascularization [14]. The present study showed that pioglitazone inhibits the expression of MMP-2, suggesting that this inhibition is involved in the mechanism by which the drug inhibits carcinogenesis in the liver. The PPARγ ligand was also reported to improve ALT levels in NASH patients [8,9], but the present study showed no significant differences between the pioglitazone and control groups in this respect. However, no significant increases in ALT levels were noted in the pioglitazone group compared to the non-pioglitazone group in rats fed with CSAA (control). Thus, pioglitazone seems to be a safe drug.

Prevention of carcinogenesis through inhibition of the progression to fatty liver and hepatic fibrosis is one of the major challenges in the treatment of NASH at present. As reported above, findings obtained in our study may indicate that pioglitazone inhibits the progression of fatty liver and hepatic fibrosis and prevents hepatocellular carcinoma in an animal model of NASH and pioglitazone can be a useful drug for the treatment of NASH.

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