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ORIGINAL PAPER

Human scavenger receptor class b type 1 is regulated by activators of peroxisome proliferators-activated receptor-γ in hepatocytes

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Abstract High-density lipoprotein (HDL) particles play a critical role in cholesterol metabolism. The hepatic scavenger receptor class B type I (SR-B1) binds HDL particles for mediating reverse cholesterol transport (RCT), thus lowering the risk of atherosclerosis. Thiazolidinediones (TZDs), known to have potent enhancing effects on insulin sensitivity, have been developed for the treatment of non-insulin-dependent diabetes mellitus. They are a high-affinity ligand for the peroxisome proliferator-activated receptor gamma (PPAR-γ), which belongs to a nuclear receptor superfamily. In this study, we examined the effects of thiazolidinedione PPAR-γ on hepatic SR-B1 gene expression in human hepatoma G2 cell-line (HepG2). Results showed that hepatic SR-B1 mRNA and protein were increased on exposure to thiazolidinediones. Transcriptional activity of human SR-B1 (hSR-B1) gene paralleled the endogenous expression of the gene and was dependent on the dose of thiazolidinediones. We investigated the influence on the promoter activity of vector expressing PPAR and retinoid X receptor (RXR), cotransfected into the HepG2 cells along with SR-B1 promoter–reporter gene constructs. PPAR-γ and RXR sufficiently induced the SR-B1 promoter activity in the HepG2 cells. Chromatin immunoprecipitation (ChIP) assay confirmed the binding of the PPAR-γ to the SR-B1 promoter region. The mutagenesis of this binding site abolished the ability of the thiazolidinediones or PPARs to stimulate promoter activity. Together, these results indicate that the stimulation of SR-B1 expression in the liver is mediated in part by activation of the PPAR-γ and RXR, and raise the possibility that this stimulation using thiazolidinediones conditions provides a protective mechanism for accelerated atherosclerosis in diabetes mellitus.

Keywords hSR-B1 · HepG2 cells · PPAR-γ · Thiazolidinediones

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Introduction

The liver is a key organ in regulating the metabolism of cholesterol. It controls the synthesis, plasma clearance, and excretion of cholesterol from the body [1, 2]. Although detailed mechanisms remain uncertain, it has been proposed that high-density lipoprotein (HDL) promotes reverse cholesterol transport (RCT) by facilitating the transfer of cholesterol from peripheral tissues to the liver to be secreted in bile [3–5]. Mouse scavenger receptor class B type I (SR-B1) mediates the selective uptake of HDL cholesterol ester (CE) into transfected Chinese hamster ovary (CHO) cells [6]. This finding provides an important link between a specific cell-surface receptor and the uptake of HDL [7–9]. Previous reports, including results from our laboratory [6, 10], showed that cells take up CE from HDL by a selective non-endocytotic pathway. Our previous studies showed that the human homologue of SR-B1 (hSR-B1), CD36 and LIMPII Analogous-1 (CLA-1), functions as a receptor for HDL, similar to the mouse homologue [6, 11–15].

Overexpression of SR-B1 in mouse liver dramatically decreases plasma HDL [16] and increases hepatic, gallbladder, and biliary cholesterol concentrations [17]. SR-B1 is a well-characterized HDL receptor that is highly expressed in the liver and in steroidogenic tissues in rodents [18]. Its human orthologue CLA-1 (hSR-B1/CLA-1) has also been shown to be a receptor for HDL [6]. Despite the fact that hSR-B1 has not been studied as extensively as rodent SR-B1, the physiological role of hSR-B1 is generally assumed to be similar to that of rodent SR-B1 [6].

Peroxisome proliferator-activated receptor gamma (PPAR-γ) is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors. It exists in two isoforms: PPAR-γ1 and the adipose tissue-restricted PPAR-γ2, which are produced from a single gene by alternative splicing and differing only by an additional 28 amino acids segment in the N-terminus of PPAR-γ2 [5, 19]. These molecules regulate gene expression by binding to DNA in a heterodimeric complex with another nuclear hormone receptor, retinoid X receptor (RXR) [20, 21]. This complex can then bind to a direct repeat consensus sequence or PPAR-γ response element (PPRE) located in the promoter region of the target genes [22, 23].

Thiazolidinediones (TZDs) represent a high-affinity ligand for PPAR-γ [22]. TZDs are known to have potent enhancing effects on insulin sensitivity, and have been developed for the treatment of non-insulin-dependent diabetes mellitus [24]. Chinetti et al. reported that hSR-B1/CLA-1 is expressed in atherosclerotic lesion macrophages and induced by PPAR activation, identifying a potential role for PPARs in cholesterol homeostasis in atherosclerotic lesion macrophages [25]. Although the role of PPAR-γ is best defined for adipogenesis and macrophage function, it is also a key regulator of cholesterol homeostasis in the liver [26]. In this study, we have examined the effect of PPAR-γ and RXR on hSR-B1/CLA-1 expression in hepatocytes.

Results

Effects of PPAR-γ ligands on hSR-B1/CLA-1 expression in HepG2 cells

To analyze the effect of TZDs on hSR-B1/CLA-1 expression, we measured the levels of endogenous hSR-B1/CLA-1 expression in HepG2 cells using Western blot analysis. Treatment of these cells with TZDs increased the abundance of endogenous hSR-B1/CLA-1 protein compared with those treated with the vehicle (Fig. 1). The maximal effect was observed at 3×10^{-6} M cilogitazone, 1×10^{-6} M troglitazone, 1×10^{-5} M piogligazone in HepG2 cells (data not shown). TZDs stimulated hSR-BI/CLA-1 expression in a time-dependent manner, we have observed the maximal effect at 24 h (data not shown). To further confirm this observation, we employed a real-time PCR to measure the effect of TZDs on hSR-B1/CLA-1 expression at the mRNA level. Results showed a similar increase in the abundance of hSR-B1/CLA-1 mRNA following treatment with TZDs; however, GAPDH expression remained unaltered (Fig. 2).

Effects of TZDs on hSR-B1/CLA-1 promoter activity

Because TZDs stimulated the abundance of both hSR-B1/CLA-1 protein and mRNA in HepG2 cells, we speculated whether TZDs regulated transcriptional activity of the hSR-B1/CLA-1 promoter in HepG2 cells. For these studies, we measured luciferase activity in HepG2 cells transfected with pCLA-LUC and exposed to TZDs (Fig. 3). In agreement with the protein and mRNA levels, treatment with TZDs also stimulated the activity of the promoter. Together, these results clearly show that TZDs increased the activity of the hSR-B1/CLA-1 gene in the HepG2 cells.

TZDs-stimulated selective CE uptake in HepG2 cells

To test the effect of TZDs-mediated stimulation of hSR-B1/CLA-1 expression on selective CE uptake in HepG2 cells, we measured the kinetics of CE uptake using doubly labeled HDL "^{125}I, 3H-HDL". Results showed that cellular HDL-CE uptake (Fig. 4) following treatment with TZDs was increased compared to the control cells. These findings support the hypothesis that treatment with TZDs stimulates the expression of hSR-B1/CLA-1 in HepG2 cells.

Effects of PPAR-γ on hSR-B1/CLA-1 promoter activity and the synergic action of RXR factor

To determine whether PPAR-γ plays a role in the TZD-induced expression of hSR-B1/CLA-1 in HepG2 cells, we transfected PPAR-γ1 and PPAR-γ2 with the hSR-B1/CLA-1 promoter to the HepG2 cells. Figure 5 shows that the luciferase activity was doubled in the case of PPAR-γ1 and PPAR-γ2, relative to the basal condition, when the pCLA-LUC construct was cotransfected with either PPAR-γ1 or PPAR-γ2 constructs. The luciferase activity showed a further 3-fold increase when the plasmid overexpressing RXRα, a heterodimer partner of PPAR-γ, was cotransfected with either PPAR-γ1 or PPAR-γ2 constructs. Treating transfected cells with 1 μM pioglitazone (PPAR-γ agonist) for 24 h increased the luciferase activity of cells cotransfected with RXRα constructs along with PPAR-γ1 or PPAR-γ2 constructs. Taken together, these data suggest that the 1.2 kb hSR-B1/CLA-1 promoter fragment may contain a PPRE (Fig. 5).

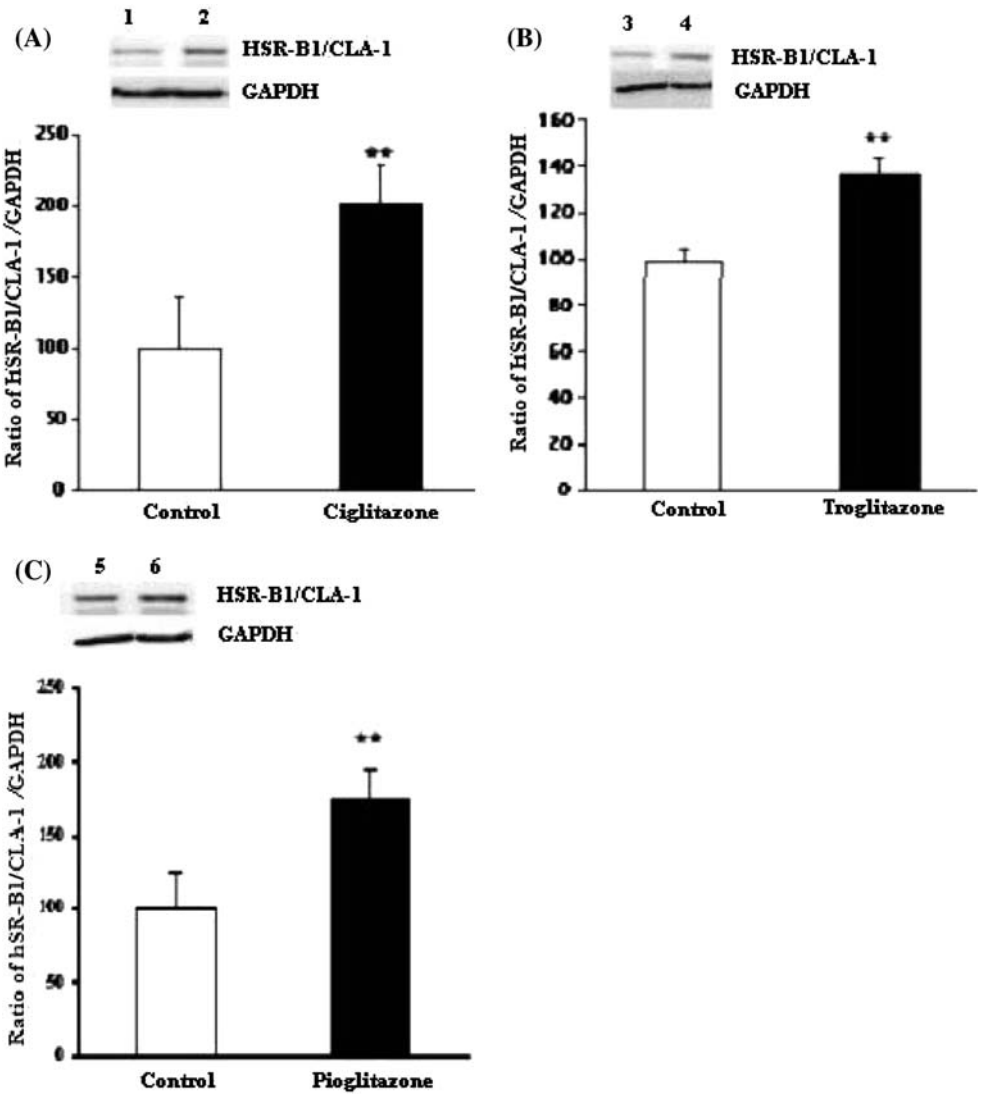

Fig. 1 TZDs increase hSR-B1/CLA-1 protein expression in HepG2 cells. The cells treated with **a** ciglitazone (3×10^{-6} M), **b** troglitazone (1×10^{-6} M), or **c** pioglitazone (1×10^{-5} M) showed increased hSR-B1/CLA-1 protein expression on Western blot analysis. Results are shown as a mean ± SEM of three experiments for each treatment group as a percentage compared to GAPDH level as a control (shown on the bottom of each lane). The *asterisk* denotes a significant difference ($P < 0.01$)

PPRE is involved in TZDs-stimulated hSR-B1/CLA-1 gene expression

Next, we searched for a DNA motif within the hSR-B1/CLA-1 promoter that may bind PPAR-γ. Examination of the promoter sequence revealed a 32-nucleotide motif, −774 5′-AGGAACGTAAGACTGGTACACGAGGTTGACCC-3′ −810, corresponding to the deduced PPRE. ChIP assay was used to determine whether PPAR-γ bound to the hSR-B1/CLA-1 promoter. Figure 6 shows the PCR amplification product after the immunoprecipitation of the cross-linked chromatin with PPAR-γ antibody (Fig. 6, lane 4). In contrast, no PCR-amplified product was found following the immunoprecipitation of the cross-linked chromatin with purified rabbit IgG (Fig. 6, lane 2). These data support the binding of PPAR-γ to hSR-B1/CLA-1 promoter that spans the nucleotides −810 to −770 on the hSR-B1/CLA-1 promoter sequence.

This finding led us to create a plasmid construct, pCLA mt-LUC, containing a mutated putative PPRE −774 5′-AGGAACGTAAGACTGGTACACGAGGTTGACCC-3′ −810 to 5′-AGGAACGTAAGACTGAGCAACGAGG TTGACCC-3′. Figure 7 shows that the luciferase activity decreased by 40%, relative to the basal condition, when hSR-B1/pCLA mt-LUC construct was cotransfected with either PPAR-γ1 or PPAR-γ2 constructs. The luciferase activity was further decreased when the plasmid over-expressing RXRα was cotransfected with either PPAR-γ1 or PPAR-γ2 constructs (Fig. 7a). Treating these transfected cells with 1 μM pioglitazone for 24 h reduced the luciferase activity (Fig. 7b). Together, these findings suggest that the putative PPRE in hSR-B1/CLA-1 promoter is involved in the TZD-mediated induction of this promoter. In addition, a mutation in the PPRE inhibited the ability of TZDs to stimulate hSR-B1/CLA-1 promoter activity. These results not only suggest that TZD-mediated

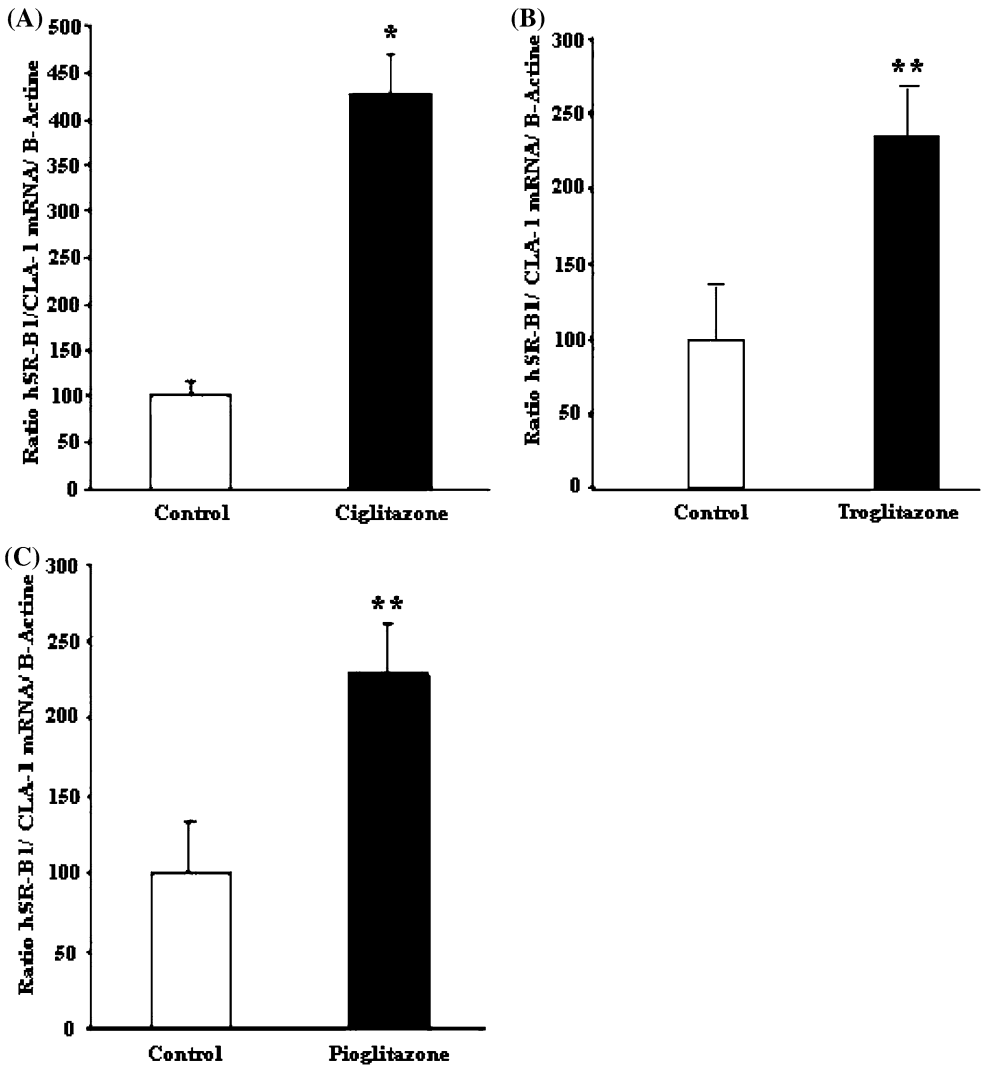

Fig. 2 TZDs increase hSR-B1/CLA-1 mRNA expression in HepG2 cells. The cells treated with **a** ciglitazone (3×10^{-6} M), **b** troglitazone (1×10^{-6} M), or **c** pioglitazone (1×10^{-5} M) showed increased hSR-B1/CLA-1 mRNA expression on real-time PCR. Results are shown as a mean ± SEM of 3 experiments for each treatment group as a percentage compared to GAPDH mRNA level as a control. The *asterisk* denotes a significant difference ($P < 0.01$)

induction of the SR-B1/CLA-1 promoter activity requires an intact PPRE motif, but also indicate the existence of the repressor element response to PPAR-γ on the hSR-B1/CLA-1 promoter.

Discussion

TZDs represent an important therapeutic option in treatment of type-2 diabetes mellitus [27]. Previous reports declared the TZDs stimulatory effect on hSR-B1/CLA-1 promoter activity in the HepG2 cells as PPAR-γ ligands [6, 10, 28]. Recently, we reported that hyperglycemia suppresses SR-B1 expression partially by the activation of the p38 MAPK-Sp1 pathway which raises the possibility that the inhibition of hepatic SR-B1 expression under high-glucose conditions provides a mechanism for accelerated atherosclerosis in diabetics [29]. Our results showed that TZDs enhanced hSR-B1/CLA-1 expression both at the protein and mRNA levels, and increased its promoter activity in HepG2 cells. These findings parallel the outcome of the previously mentioned studies.

Clinical research data demonstrate that TZDs also protect against the development of atherosclerosis by preventing the formation of foam cells, lowering blood pressure, limiting the inflammatory response, and by affecting the lipid profile [30]. Regotti et al. [31] reported higher plasma cholesterol concentrations in mice with a null mutant SR-B1 gene due to decreased selective cholesterol uptake. Also, previous studies showed that feeding APOE-deficient mice with BRL49653 or GW2331, a combined PPAR-α and PPAR-γ agonist, increased cholesterol efflux from macrophages and decreased atherosclerotic areas with no change in HDL cholesterol levels, indicating that the increased HDL-associated cholesterol was rapidly cleared from circulation by the liver, possibly by hepatic SR-B1 [32, 33]. Consequently, SR-B1 is believed to act as a docking receptor for HDL [34]. The

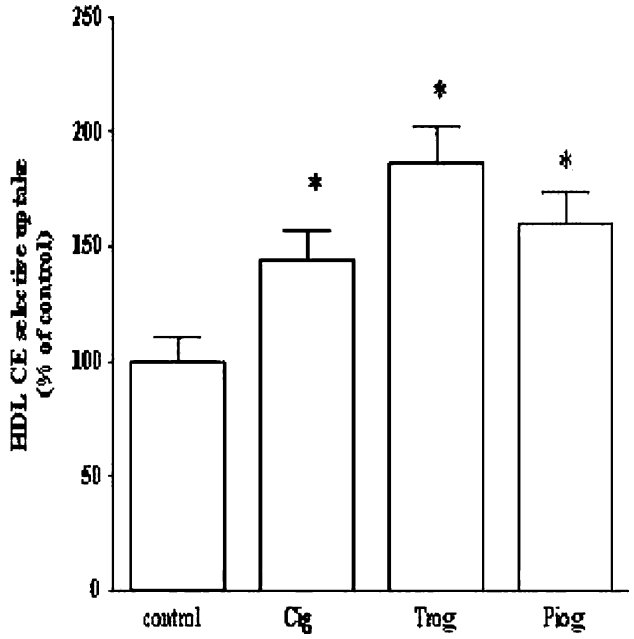

Fig. 3 TZDs increase selective HDL-CE uptake in HepG2 cells. The cells treated with ciglitazone (Cig; 3×10^{-6} M), troglitazone (Trog; 1×10^{-6} M), and pioglitazone (Piog; 1×10^{-5} M) for 24 h and incubated with 10 μg/ml of (^{125}I, 3H)-HDL showed an increased HDL-CE selective uptake. Values represent the mean of three experiments. The *asterisk* denotes a significant difference ($P < 0.01$)

influence of hepatic SR-B1 expression on macrophage RCT may help to explain its effect on atherosclerosis, and to date, serves as the clearest demonstration of the principle that steady-state plasma concentrations of HDL-C are not necessarily predictive of the rate of macrophage RCT [35]. The aforementioned reports support our study which showed an increased selective CE uptake on treating HepG2 cells by TZDs through increasing SR-B1 expression.

In the consecutive course of our studies, we found that PPAR-γ isoforms, PPAR-γ1 and the adipose tissue-restricted PPAR-γ2 enhance SR-B1/CLA-1 expression. This effect was further enhanced by cell transfection with RXR factor (synergic effect), while it was further potentiated by introducing TZDs (Piog) showing higher SR-B1 expression levels. Furthermore, ChIP assay declared the binding site of PPAR-γ to SR-B1/CLA-1 promoter. On the contrary, transfecting HepG2 cells with a mutant SR-B1/CLA-1 gene abolished these results. These findings are in agreement with the previous reports which concluded that the stimulatory effects of TZDs on hSR-B1/CLA-1 promoter activity in the HepG2 cells require the participation of the transcriptional factors PPAR-γ and RXR [28]. The PPAR-γ/RXR receptor dimer is involved in the transcriptional control of energy, lipid, and glucose homeostasis [36, 37]. The increase in reporter gene activity after overexpression of the RXR gene in HepG2 cells may be due to homodimer formation. Indeed, a recent study has demonstrated, using in vivo ChIP assay, that overexpressed RXR homodimers can selectively bind to functional PPREs and induce transactivation [38]. This further strengthens our findings which support the role of PPAR-γ and its ligands (TZDs) in regulation of SR-B1/CLA-1 expression.

In summary, the results of this study show that TZDs stimulates hepatic endogenous hSR-B1 expression. This stimulatory effect of TZDs on hSR-B1 promoter activity is mediated by PPAR-γ/RXR. These findings raise the possibility that TZDs may affect RTC by controlling hSR-B1/CLA-1 expression and may have a protective therapeutic role in atherosclerosis.

Materials and methods

Reagents

Thiazolidinedione, ciglitazone, troglitazone, and pioglitazone were purchased from Cayman Chemical (Ann Arbor, MI). An anti-PPAR-γ antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cells

Human hepatoma HepG2 cell-line (obtained from RIKEN Cell Bank, Ibaraki, Japan) was grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Life Technologies, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). Human umbilical vein endothelial cells (HUVEC) were cultured as described in an earlier study [39].

Antibodies

To create an antibody directed against the extracellular domain (residues 185–300) of hSR-B1/CLA-1 [40], the corresponding complementary DNA (cDNA) fragment was amplified from human monocyte-derived THP-1 cells (American Type Culture Collection (ATCC), Manassas, VA) using polymerase chain reaction (PCR). The product of this reaction was inserted into the vector pGEX-2T (Pharmacia Biotech, Uppsala, Sweden). The nucleotide sequence was verified and the peptide was expressed in *Escherichia coli*. The peptide of interest fused to glutathione S-transferase (GST) was isolated using glutathione Sepharose™ 4B beads (Pharmacia). The bound material was injected into guinea pigs to generate antiserums against hSR-B1/CLA-1. Western blot analysis of proteins extracted from the cells stably expressing hSR-B1/CLA-1 showed that the antibody was directed against an

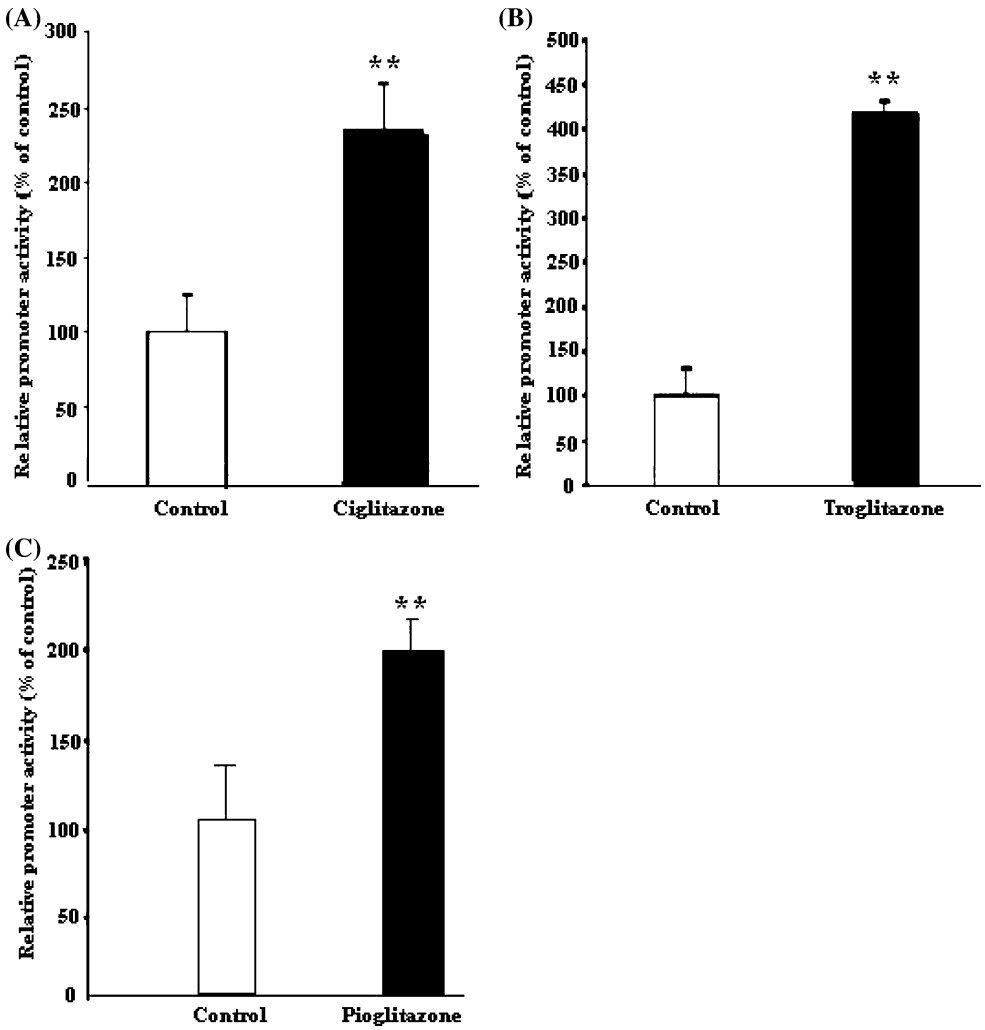

Fig. 4 TZDs increase the hSR-B1/CLA-1 promoter expression in HepG2 cells. The cells transfected with pCLA-LUC then treated with **a** ciglitazone (3×10^{-6} M), **b** troglitazone (1×10^{-6} M), or **c** pioglitazone (1×10^{-5} M) showed an increased hSR-B1/CLA-1 promoter expression. Results are expressed as relative luciferase activity compared to that of control cells, arbitrarily set at 100. Each data point shows the mean ± SEM of three separate transfections, which were performed on separate days. The *asterisk* denotes a significant difference ($P < 0.01$ or 0.05)

extracellular portion of the protein, as was described in an earlier study [6].

Protein extraction and Western blot analysis

HepG2 cells were treated with pioglitazone, ciglitazone, and troglitazone for 24 h; washed with phosphate buffered saline (PBS); and lysed in PIRA buffer (10 mM Tris-HCl (pH 7.4), 1% Tergitol-type NP-40 (NP40), 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 0.15 M NaCL, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 μg/ml aprotinin). The proteins were resuspended under reducing conditions, and 15 μg was fractionated by size on 7.5% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes for immunoblotting. The membranes were blocked overnight at room temperature with 0.1% Tween-20 in PBS (PBS-T) containing anti-hSR-B1/CLA-1 antibody (diluted 1:3000 from whole antiserum) or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Biomol Research, Plymouth Meeting, PA; diluted 1:1000). These membranes were washed with PBS-T, incubated for 1 h at 4°C in PBS-T containing horseradish peroxidase-linked anti-guinea pigs IgG (diluted 1:3000) and antibody binding was visualized by enhanced chemiluminescence detection (ECL; Amersham Corp., Arlington Heights, IL).

Real-time reverse transcriptase PCR

PCRs were performed using a final volume of 20 μl in LightCycler® (Roche, Mannheim, Germany) glass capillaries. The reaction mixture comprised 2 μl LightCycler Fast-Start DNA Master SYBR Green-I (Roche), 2.4 μl 25 mM $MgCl_2$ stock solution, 11.6 μl sterile PCR-grade water, 2 μl of the cDNA template for each gene of interest, and 1 μl of 10 μM of each primer. The sequences of the forward and reverse hSR-B1/CLA-1 primers were 5′-TGG GGATGCCTTCAAACAC-3′ and 5′-TTGAACTTCTG GGCAAATG-3′, respectively. The cycling program consisted of initial denaturation for 60 s at 95°C followed by 55 cycles of 95°C for 5 s, 62°C for 5 s, and 72°C for 15 s, with a 20°C/s slope. Each set of reactions included water as

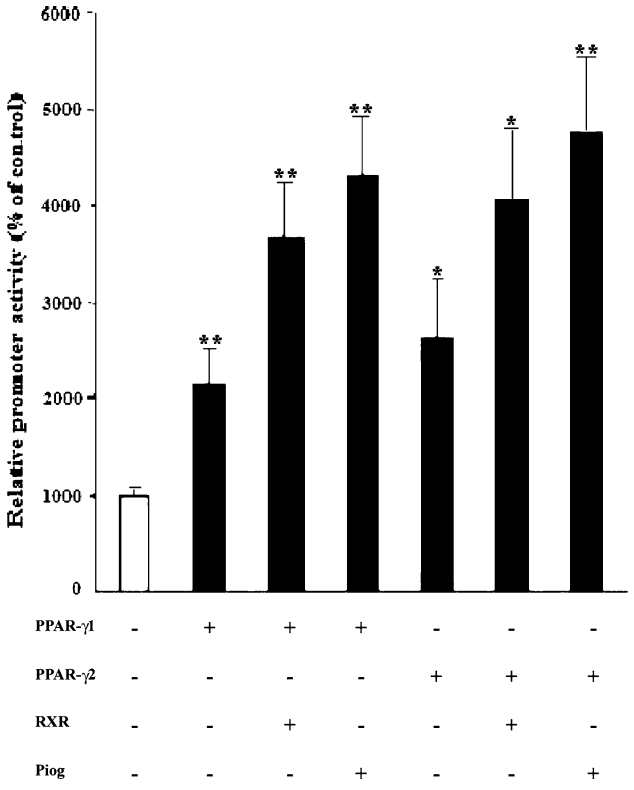

Fig. 5 PPAR-γ1 and PPAR-γ2 increase hSR-B1/CLA-1 promoter expression in HepG2 cell. The cells transfected with pCLA-LUC then with either PPAR-γ1 or PPAR-γ2 expression vector showed an increased hSR-B1/CLA-1 promoter expression. Transfection with RXR factor in both groups of cells showed a higher level of hSR-B1/CLA-1 promoter expression (synergic effect). Treatment of both groups with pioglitazone (1×10^{-5} M) further increased hSR-B1/CLA-1 promoter expression. Each data point shows the mean ± SEM of four different transfections, which were performed on separate days. The *asterisk* denotes a significant difference ($P \leq 0.01$ or 0.05)

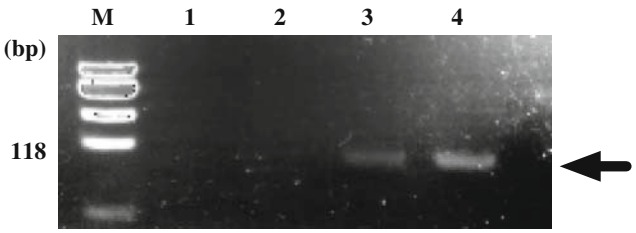

Fig. 6 ChIP assay demonstrating the binding site of PPAR-γ to CLA-1 promoter. The PCR product was observed in the anti-RNA Polymerase-II ChIP (lane 4), but not in the normal mouse IgG ChIP (lane 2). CLA-1 specific DNA was also observed in the Input (lane 3), but not in the "no DNA" PCR control (lane 1). An identical experiment that was performed independently showed similar results

a negative control and five dilutions of standard. Known amounts of DNA were then diluted to provide standards and a regression curve of crossing points versus concentration generated with the LightCycler. GAPDH was used as a standard, as described in an earlier study [15].

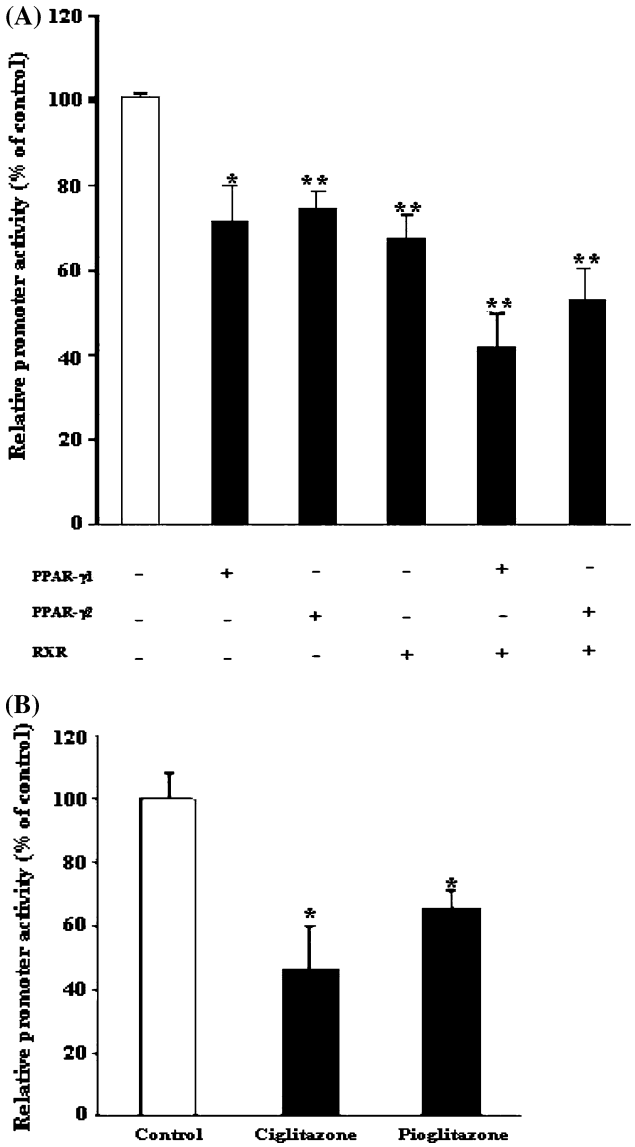

Fig. 7 **a** Mutant hSR-B1/CLA-1 promoter abolishes the effect of PPAR-γ1 and PPAR-γ2 in HepG2 cells. Also abolished the synergic effect of RXR with PPAR-γ1 and PPAR-γ2. **b** Mutant hSR-B1/CLA-1 promoter abolishes the enhancing effect of ciglitazone (10^{-6} M) and pioglitazone (1×10^{-5} M) in HepG2 cells. The results were expressed as relative luciferase activity compared with control cells arbitrarily set at 100. Each data point shows the mean ± SEM of three separate transfections that were performed on separate days. The *asterisk* denotes a significant difference ($P < 0.01$)

Transfection of HepG2 cells and luciferase reporter gene assay

The reporter construct contained the hSR-B1/CLA-1 gene sequence spanning the region from −1200 to +2, as determined from an earlier published sequence [41]. Purified reporter plasmid was transfected into HepG2 cells (at 60% confluence) using a conventional cationic liposome transfection method (Lipofectamine, Life Technologies,

Gaithersburg, MD). All assays were corrected for β-galactosidase activity and the total amount of protein in each reaction was identical. Aliquots of 20 µl were taken for the luciferase assay, which was performed according to the manufacturer's instructions (ToyoInk, Tokyo, Japan).

HDL-selective CE uptake

Human HDL ($d = 1.070$–1.20 g/ml) was isolated by preparative ultracentrifugation from fresh plasma collected in EDTA (1 mg/ml), as described in an earlier study [42]. HDL was passed through a heparin–Sepharose affinity column to remove particles containing apolipoprotein E (APOE) [43]. The isolated lipoproteins were iodinated by the Iodogen method (Pierce, Rockford, IL) to a specific activity of 400–600 cpm/ng protein for HDL. For specific uptake studies, the lipid moiety of human HDL was labeled with (^{3}H) cholesterol ester ((^{3}H) CE) and the apolipoprotein A-I (APOA-I) with ^{125}I. The former ((^{3}H) CE) was prepared from (^{3}H) cholesterol and oleic anhydride, as previously described [10]. Human APOA-I was purified from HDL, labeled with ^{125}I, and then exchanged into the (^{3}H) HDL. HepG2 cells were treated with ciglitazone (3×10^{-6} M), troglitazone (1×10^{-6} M), or pioglitazone (1×10^{-5} M) for 24 h were incubated at 37°C for 1.5 h with 10 µg/ml of [125I, 3H]-HDL followed by processing to determine HDL-CE selective uptake. The values for the selective uptake of HDL-CE were obtained as described in a previous study [6].

Plasmid preparation

The reporter construct contained the hSR-B1/CLA-1 gene sequence spanning the region from −1200 to +2, as determined from a published sequence [42]. The segment of interest was amplified using PCR and cloned into the luciferase reporter gene (pCLA-LUC). To generate the mutant construct (pCLA mt-LUC) of the PPRE within the vector, pCLA-LUC was mutated from (−774) 5′-AGGA ACGTAAGACTGGTACACGAGGTTGACCC-3′ (−810) to 5′-AGGAACGTAAGACTGAGCAACGAGGTTGACC C-3′ (mutated nucleotides are underlined) by site-directed mutagenesis, as previously reported [44].

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed using EZ-ChIP™ kit (Upstate, Charlottesville, VA) according to the manufacturer's protocol. To cross-link proteins to DNA, formaldehyde (final concentration 1%) was added to the culture medium and incubated for 10 min at room temperature. Then, a final concentration of 0.125 M glycine was added to stop fixation, and cells were scraped and collected by centrifugation at 700 × *g* for 5 min at 4°C. Cell pellets were treated with SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris; pH 8.1) containing protease inhibitors. Aliquots of cell lysates were sonicated to shear DNA into 0.2–1.0 kb fragments and cellular debris was removed by centrifugation at 14,000 × *g* for 15 min at 4°C. The resultant chromatin-containing solutions were aliquoted (100 µl) and stored at −80°C until use. Chromatin aliquots were pre-cleared with 60 µl of 50% protein G agarose suspension. Samples were then incubated with anti-PPAR-γ antibody (Santa Cruz Biotechnologies) or normal rabbit IgG (as a control; Santa Cruz Biotechnologies) overnight at 4°C with rotation. Immunocomplexes were mixed with 60 µl of 50% protein G agarose suspension followed by incubation for 1 h at 4°C with rotation. Beads were collected by brief centrifugation and the immunocomplexes were eluted by freshly prepared elution buffer (100 mM $NaHCO_3$, 1% SDS). Chromatin was then de-cross-linked for 5 h at 65°C. After treatment with proteinase K, DNA was purified with QIA quick PCR Purification Kits (Qiagen, Valencia, CA) and finally eluted in 50 µl of TE (Tris–EDTA) buffer. An aliquot (2 µl) of each sample was subjected to PCR analysis using HotStar Taq DNA polymerase (Qiagen; 32 cycles).

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