Upregulation of hepatic LRP1 by rosiglitazone: a possible novel mechanism of the beneficial effect of thiazolidinediones on atherogenic dyslipidemia

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Abstract

Hepatic LDL receptor-related protein 1 (LRP1) plays a role in the clearance of circulating remnant lipoproteins. In this study, we investigated the effect of rosiglitazone treatment on the expression and function of hepatic LRP1. HepG2 cells were incubated with various concentrations of rosiglitazone. Male Long-Evans Tokushima Otsuka (LETO) rats and Otsuka-Long-Evans-Tokushima Fatty (OLETF) rats were treated with rosiglitazone for 5 weeks. The expression and function of LRP1 in HepG2 cells and liver samples of rats were analyzed. LRP1 mRNA and protein expressions were increased by 0.5 and 5 μ M rosiglitazone in HepG2 cells. However, at concentrations above 50 μ M rosiglitazone, LRP1 mRNA and protein expressions did not change compared with those in nontreated cells. Reporter assay showed that 0.5 and 5 μ M rosiglitazone increased the transcriptional activity of the *LRP1* promoter in HepG2 cells. The uptake of apolipoprotein E through LRP1 in HepG2 cells was also increased by rosiglitazone. Hepatic LRP1 was reduced in OLETF rats compared with that of LETO rats and rosiglitazone treatment increased hepatic LRP1 in OLETF rats. A high glucose condition (25 mM glucose in culture media) reduced the expression of LRP1 in HepG2 cells, and this reduced LRP1 expression was recovered with rosiglitazone. In conclusion, our data suggest that decreased hepatic LRP1 in a diabetic condition is associated with the development of atherogenic dyslipidemia and that increased hepatic LRP1 by thiazolidinediones could contribute to an improvement in atherogenic lipid profiles in diabetic patients.

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Introduction

Diabetes mellitus (DM) is a major risk factor for cardiovascular disease. DM can cause atherogenic dyslipidemia including elevated triglycerides and low HDL cholesterol (HDL-C; Gordon *et al.* 1989, Assmann & Schulte 1992). Increased free fatty acid due to insulin resistance in adipose tissue promotes the hepatic production of triglycerides, which are packaged in apolipoprotein B-containing VLDLs (Bell *et al.* 2011). Increased triglyceride-rich lipoproteins, such as chylomicron and VLDL, are hydrolyzed to remnant particles (Merkel 2009). Recent studies have focused on these remnant lipoproteins as atherogenic particles, showing that remnant lipoproteins can penetrate the endothelial wall and remain in the subendothelial space (Fujioka & Ishikawa 2009). Remnant lipoproteins have also been reported to be associated with inflammation,

endothelial dysfunction, foam cell formation, and vascular smooth muscle cell proliferation (Fujioka & Ishikawa 2009). Several studies have reported that the level of remnant-like particle cholesterol (RLP-C) is increased in diabetic patients (Taniguchi *et al.* 2000, Schaefer *et al.* 2002) and is associated with an increased risk of cardiovascular disease (Fukushima *et al.* 2001, 2004).

LDL receptor-related protein 1 (LRP1) is a member of the LDD receptor gene family and is a multi-functional scavenger and signaling receptor that binds and internalizes diverse ligands (Herz & Strickland 2001). This cell surface glycoprotein binds apolipoprotein E (ApoE) and serves as a receptor for remnant lipoproteins such as chylomicron remnant and VLDL remnant in the liver (Herz & Strickland 2001). Furthermore, this receptor plays an important role in the clearance of remnant lipoproteins

(Willnow *et al.* 1994, 1995, Rohlmann *et al.* 1998). A thiazolidinedione drug, such as rosiglitazone or pioglitazone, is an oral hypoglycemic agent that improves insulin resistance by activating the peroxisome proliferator-activated receptor- γ (PPAR γ). This agent has also been known to modify the atherogenic lipid profile. In clinical studies, pioglitazone decreased the serum level of triglycerides and RLP-C and increased serum HDL-C (Goldberg *et al.* 2005, Berhanu *et al.* 2006, Berneis *et al.* 2008, Igarashi *et al.* 2008). Rosiglitazone also decreased serum triglycerides in some animal and clinical studies (van Wijk *et al.* 2005, Ackerman *et al.* 2010, Yang *et al.* 2011). Pioglitazone has been reported to reduce serum triglycerides by increasing their clearance from the circulation, most likely through increased lipoprotein lipase-mediated lipolysis of VLDLs (Hanefeld 2009). One *in vitro* study reported that LRP1 is regulated by PPAR γ and that rosiglitazone induced LRP1 expression in human adipocytes (Gauthier *et al.* 2003). On the basis of these data, we hypothesized that thiazolidinediones may increase hepatic LRP1 and the hepatic clearance of remnant lipoproteins. In this study, the effect of rosiglitazone on the expression and function of hepatic LRP1 was investigated. Our study may present a novel mechanism of the improvement of atherogenic dyslipidemia by a thiazolidinedione drug in diabetic patients.

Materials and methods

Cell culture and preparation

The HepG2 cells were cultured in MEM containing 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 mg/ml) at 5% CO₂/95% air and 37 °C. Rosiglitazone was provided by GlaxoSmithKline and prepared in DMSO at -20 °C. HepG2 cells were incubated with the indicated concentrations of rosiglitazone for 48 h by adding the stock solution to the culture media. The final concentration of DMSO in the culture media was adjusted to 0.5% (vol/vol).

Animals, diet, and treatment

Laboratory animals for all experiments were cared for in accordance with the National Institute of Health's guidelines. The animals were maintained according to the ethical guidelines of our institution, and the experimental protocol was approved by the Committee on Animal Investigations of Yonsei University. Male Otsuka-Long-Evans-Tokushima Fatty (OLETF) rats (Tokushima Research Institute, Otsuka Pharmaceuticals, Tokushima, Japan) were used as a diabetic animal model and Male Long-Evans Tokushima

Otsuka (LETO) rats (Tokushima Research Institute, Otsuka Pharmaceuticals) were used as nondiabetic counterparts. The rats were housed in a temperature-controlled environment under a 12 h light:12 h darkness cycle and allowed *ad libitum* access to standard chow and water.

All rats were fed standard chow until the age of 20 weeks. Thereafter, OLETF rats were fed a high-fat diet (40% lard, Wellga, Inc., Seoul, Korea). OLETF rats were randomly divided into two groups: the vehicle (normal saline)-treated group ($n=9$) and the rosiglitazone-treated group ($n=8$). The rosiglitazone-treated rats were administered 4 mg/kg per day compound in saline via oral gavage using 20 G feeding needles. LETO rats received standard chow without any drug ($n=8$). All the animals were treated for 5 weeks. One day after the OGTT at the age of 25 weeks, all the rats were killed; the livers were extracted, processed, and embedded in paraffin for histological analysis. The remaining tissues were flash-frozen in liquid nitrogen and stored at -80 °C until analysis. Blood was collected by cardiac puncture and stored at -20 °C for biochemical tests.

Oral glucose tolerance test

All the rats underwent an OGTT after an overnight fast using 20% glucose solution (2 g/kg). Blood samples were obtained by tail snipping, and blood glucose levels were measured with a glucose analyzer (Accu-Check; Roche Diagnostics). Glucose levels were recorded at 0, 15, 30, 60, and 120 min after glucose administration.

Biochemical analysis

Blood samples were obtained from the heart at the time of killing and were immediately centrifuged at 5000 *g* for 5 min. Total cholesterol and triglycerides were determined using an ADVIA 1650 (Bayer).

Total RNA and cDNA preparation

Total RNA was isolated from HepG2 cells and the rat liver tissues using Trizol reagent (Invitrogen) and was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Rockford, IL, USA). Following the RNA extraction, 4 μ g RNA were treated with 1 U RNase-free DNase I to remove all contaminating genomic DNA. After removing the DNase I, DNase-treated RNA was subsequently used for cDNA synthesis using MMLV reverse transcriptase (Promega) according to the manufacturer's protocol. The synthesized cDNA was stored at -20 °C for later use.

Quantitative real-time PCR

Quantitative real-time PCR (RT-PCR) analysis was performed using TaqMan assay kits for LRP1 (Hs00233856_m1, R701503964 g1) with the ABI 7500 instrument (Applied Biosystems). The β -actin (Hs99999903_m1, R700667669_m1) gene was used as an invariant control. PCRs were carried out in triplicate reactions in a final volume of 20 μ l according to the manufacturer's protocol. For each assay, a standard curve was obtained by analyzing a series of dilutions of pooled cDNA samples for the relevant gene. Data were analyzed with Sequence Detector 1.7 software (Applied Biosystems). β -Actin was used as an invariant control and the results were expressed as a ratio of the gene expression relative to that of β -actin.

Immunoblot analysis

Cell lysates were prepared using MPER (Thermo Scientific) and aliquots of cell lysates and tissue homogenates were denatured under reducing conditions (1.75% SDS, 15 mM 2-mercaptoethanol) for 5 min at 100 °C. The total protein amount in each cell lysate was determined by Bradford assay (Sigma–Aldrich). Cell lysates including 10 μ g protein were loaded to SDS–PAGE for immunoblot analysis. For LRP1, nitrocellulose membranes were incubated with anti-LRP1 antibody (Epitomics, Burlingame, CA, USA) at a 1:1500 dilution overnight at 4 °C and then subsequently with HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) at a 1:5000 dilution for 1 h at room temperature. The signals were detected with the ECL Western Blotting Analysis System (Thermo Scientific). β -Actin immunoreactivity, which detected with monoclonal anti- β -actin antibody (Sigma–Aldrich) at a 1:5000 dilution and HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) at an 1:5000 dilution, was used as a loading control.

Immunohistochemistry

Rat livers were paraformaldehyde fixed, paraffin embedded, and cut into 5–6 mm sections. The sections were stained with an antibody to LRP1 (Epitomics, 1:100). Staining was visualized using a 3,30-diaminobenzidine tetrahydrochloride (DAB) kit (Evision plus kit, Dako, Glostrup, Denmark).

Electromobility shift assay

The nuclear protein extracts of HepG2 cells were prepared using the Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). Double-stranded oligonucleotides of a peroxisome

proliferator response element (PPRE) in human LRP1 promoter (5'-CCCCGCTCCTTGAACCTCTGACATGCAGACACCTA-3') were synthesized and end labeled with biotin (Gauthier *et al.* 2003). Also, the oligonucleotides of the mutated form of the LRP1 PPRE (5'-CCCCGCTCCTTGAACCTCAACGATGCAGACACCTA-3') were synthesized and biotin labeled (Gauthier *et al.* 2003). Nuclear protein extracts were incubated with the labeled oligonucleotides in the presence of poly(dI-dC) in a binding buffer containing 10 mM Tris–HCl, 5 mM MgCl₂, 0.5 mM dithiothreitol, 5 mM EDTA, 5% glycerol, and 50 mM KCl at room temperature for 20 min. DNA–protein complexes were then resolved by electrophoresis on 10% polyacrylamide gels and visualized by the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific).

For the supershift assays, co-incubation was performed using 0.5 μ g anti-PPAR γ antibody (R&D Systems, Minneapolis, MN, USA) before adding the labeled oligonucleotides. The reaction was carried out at 4 °C for 30 min. To examine the specificity, a 200-fold excess of unlabeled double-stranded oligonucleotides was added as a competitor in the binding reaction.

Construction of the LRP1 promoter-reporter vector

The LRP1 promoter-reporter was constructed according to the method previously reported by Gauthier *et al.* (2003). The promoter region spanning the upstream 1.9 kb of the 5'-flanking region of human LRP1 that contains a PPRE was amplified by PCR using the LRP1-BAC construct as a template (Source BioScience imaGenes, Berlin, Germany) using the following primers: 5'-GCAACGAGCTCCGTAAAAGG-GGGAAG-3' and 5'-GCAGCAGATCTTCCCCGGACT-GAAG-3'. The amplified fragment was subcloned into the SacI and BglII sites of the firefly luciferase reporter vector, pGL3-Basic (Promega), and designated as pGL3-PPRE. The integrity of the reporter plasmid sequences was confirmed by DNA sequencing.

Transient transfection assays

HepG2 cells were seeded at a density of 1.5×10^5 cells/well in six-well plates 48 h before transfection. When the cell density reached a confluency of 90%, cells were co-transfected with 4 μ g pGL3-PPRE and 0.25 μ g pRL-CMV, the Renilla luciferase reporter vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The pGL3-Basic vector was used for the negative control. Four hours after the transfection, the cells were treated with the indicated concentrations of rosiglitazone for 48 h. The cell lysates were prepared with 250 μ l reporter lysis buffer (Promega).

Luciferase activities derived from both firefly (pGL3-PPRE) and Renilla (pRL-CMV) proteins were measured using the dual luciferase reporter assay system (Promega) using a Berthold luminometer (Berthold, Wildbad, Germany). The firefly luciferase activity was normalized with Renilla luciferase activity to minimize any experimental variability caused by differences in cell viability or transfection efficiency.

siRNA transfections

siRNA targeting human LRP1 (siLRP1) and non-targeting negative siRNA (siCTRL) were purchased from Thermo Scientific. Each siRNA was transfected into HepG2 cells using Lipofectamine 2000 (Invitrogen). We determined siRNA silencing efficiency by RT-PCR of *LRP1* mRNA in siLRP1- and siCTRL-transfected HepG2 cells.

ApoE uptake analysis

HepG2 cells were treated with the indicated concentrations of rosiglitazone. If needed, siLRP1 was transfected to HepG2 cells for *LRP1* gene silencing before rosiglitazone treatment. After 48 h, cells were washed once with PBS and then incubated with 25 μ g/ml human recombinant ApoE3 (R&D Systems) for 1 h. ApoE3 was reconstituted with lipid using 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) before the treatment on HepG2 cells using the method of previous studies (Innerarity *et al.* 1979). For further analysis, HepG2 cells were washed three times with PBS and harvested with Mammalian Protein Extraction Reagent (Thermo Scientific). The lysates were subjected to SDS-PAGE using anti-human ApoE3 antibody at a 1:150 000 dilution (R&D Systems).

Statistical analysis

All statistical analyses were conducted using SPSS software (version 18.0; SPSS, Chicago, IL, USA). Values are expressed as the mean $\pm$ s.e.m. Statistical comparisons between groups were performed using the Student's *t*-test. Data with a *P* value <0.05 were considered significant.

Results

Rosiglitazone increased LRP1 expression in HepG2 cells in a dose-dependent manner

In HepG2 cells, a human hepatoma-derived cell line, quantitative RT-PCR and immunoblotting showed that *LRP1* mRNA and protein expressions were increased by 0.5 and 5 μ M rosiglitazone (Fig. 1A and B). However, at

Figure 1 The effect of rosiglitazone on *LRP1* expression in HepG2 cells. HepG2 cells were treated with the indicated concentrations of rosiglitazone for 48 h. (A) RT-PCR quantification of *LRP1* mRNA in HepG2 cells ($n=6$ /group). (B) Western blot analysis of LRP1 (β -chain) in HepG2 cells ($n=6$ /group). (C) Western blot analysis of LRP1 (β -chain) in HepG2 cells. Results were normalized to β -actin mRNA or protein level and are expressed as ratios relative to the nontreated cells. Data are mean $\pm$ s.e.m. * $P<0.05$ vs rosiglitazone 0 nM.

concentrations above 50 μ M rosiglitazone, *LRP1* mRNA and protein expressions did not change compared with those in the nontreated cells (Fig. 1A and B). We confirmed that rosiglitazone at concentrations up to 5 μ M increased the protein amount of LRP1 in HepG2 cells in a dose-dependent manner (Fig. 1C).

Rosiglitazone induced the binding of PPAR γ to a PPRE of the *LRP1* promoter in HepG2 cells

To demonstrate the mechanism of LRP1 upregulation by rosiglitazone, an electromobility shift assay (EMSA) was performed on the oligomers corresponding to the PPRE sequence of the *LRP1* promoter. The conserved PPRE sequence located at -1185 to -1173 of the human *LRP1* promoter (Gauthier *et al.* 2003). The results presented in Fig. 2A showed an increased gel-retarded band in samples derived from the HepG2 cells treated with 0.5 μ M rosiglitazone (lanes 1 and 2). The supershift of this gel-retarded band by the anti-PPAR γ antibody supports the involvement of PPAR γ in the protein-DNA complex (lane 3). The disappearance of a gel-retarded band in the assays using the excessive unlabeled competitor oligomer and the mutated oligomer shows the involvement of the PPRE sequence of the *LRP1* promoter in the protein-DNA complex (lanes 4 and 5).

Rosiglitazone increased the *LRP1* promoter activity in HepG2 cells

The transcription activity of the *LRP1* promoter in HepG2 cells was assayed using the promoter-reporter construct. The promoter-reporter construct contains the *LRP1* promoter region including a conserved PPRE. The reporter assay in HepG2 cells showed that rosiglitazone increased the transcriptional activity of the *LRP1* promoter at concentrations up to 5 μ M (Fig. 2B). However, the *LRP1* promoter activity in HepG2 cells remained unchanged at rosiglitazone concentrations above 50 μ M (Fig. 2B).

Rosiglitazone increased the uptake of ApoE in HepG2 cells

We performed an ApoE uptake analysis in HepG2 cells to investigate the function of LRP1 increased by rosiglitazone. After the incubation of HepG2 cells with various concentrations of rosiglitazone for 48 h, the cellular uptake of ApoE during 1 h was measured. We confirmed that the expression of endogenous ApoE was not affected by rosiglitazone treatment in HepG2 cells (Fig. 3A). The uptake of ApoE in HepG2 cells was increased by rosiglitazone treatment (Fig. 3A). The ApoE uptake in HepG2 cells was not increased

A

TGACCTnTGACCT	Consensus PPRE
TGAACCTGACAT	PPRE of <i>LRP1</i> promoter (83%)
TGAACCAACCAT	Mutated form of the <i>LRP1</i> PPRE

B

Figure 2 The effect of rosiglitazone on the *LRP1* promoter activity in HepG2 cells. HepG2 cells were treated with the indicated concentrations of rosiglitazone for 48 h. (A) EMSA was performed on oligomers corresponding to a PPRE of the *LRP1* promoter. Nuclear extracts were prepared from HepG2 cells without rosiglitazone treatment (lane 1) or with 0.5 μ M rosiglitazone (lanes 2–5). Anti-PPAR γ antibody was co-incubated before adding the labeled oligomers for a supershift assay (lane 3). Unlabeled oligomers of the *LRP1* PPRE at 200-fold molar excess were used to compete with labeled oligomers (lane 4). Labeled oligomers of the mutated form of the *LRP1* PPRE were used for specification (lane 5). (B) The promoter region of human *LRP1* was cloned into pGL3-basic. This construct was transiently co-transfected into HepG2 cells along with the Renilla luciferase reporter vector, pRL-CMV. For the negative control, pGL3-basic vector was used. Firefly and Renilla luciferase activities were determined using the dual luciferase assay system. The relative luciferase activities were calculated as the ratio of normalized luciferase activities in cells treated with the indicated concentrations of rosiglitazone relative to that of cells without rosiglitazone treatment. Data are mean $\pm$ S.E.M. of three independent transfection experiments (each in triplicate reactions). * P < 0.05 vs rosiglitazone, 0 nM.

Figure 3 The effect of rosiglitazone on ApoE uptake in HepG2 cells. HepG2 cells were treated with indicated concentrations of rosiglitazone for 48 h. Human recombinant ApoE3 was added to culture media and cells were incubated for 1 h. ApoE3 was reconstituted with lipid using DMPC before the treatment on HepG2 cells. Three independent experiments were performed for the representative figures. (A) Western blot analysis of ApoE3 in HepG2 cells incubated with or without added ApoE3. (B) Western blot analysis of ApoE3 in HepG2 cells incubated with added ApoE3. HepG2 cells were transfected with siRNA targeting human LRP1 (siLRP1) and non-targeting negative siRNA (siCTRL) before the rosiglitazone treatment and adding ApoE3.

by rosiglitazone when *Lrp1* gene was knocked down by siRNA (Fig. 3B). The increase in ApoE3 uptake by rosiglitazone in HepG2 cells is specifically through the increase in LRP1. The transfection efficiency, which was determined by RT-PCR of LRP1 in siRNA-transfected HepG2 cells, was $72.9 \pm 4.7\%$.

LRP1 expression was decreased in the high-fat-fed OLETF rat livers and was recovered by rosiglitazone treatment

To confirm the effect of rosiglitazone on hepatic LRP1 expression *in vivo*, we compared hepatic LRP1 expression among the three experimental animal groups: LETO rats fed a standard chow diet, OLETF rats fed a high-fat diet, and high-fat-fed OLETF rats treated with rosiglitazone (4 mg/kg per day for 5 weeks). The results of the OGTT showed the development of DM in high-fat-fed OLETF rats and the improvement in glycemic control in rats treated with rosiglitazone (Fig. 4A). The serum triglyceride level was increased in high-fat-fed OLETF rats in comparison to the level in LETO rats and was partially recovered by rosiglitazone treatment (Fig. 4B). Interestingly, immunoblotting and immunohistochemistry showed that the protein expression of hepatic LRP1 was reduced in high-fat-fed OLETF rats compared with that of LETO rats, and the rosiglitazone treatment increased the protein expression of hepatic LRP1 in OLETF rats (Figs 5A and 6).

LRP1 expression in HepG2 cells was decreased under a high glucose condition and recovered by rosiglitazone treatment

To elucidate the factors that decrease the hepatic LRP1 expression, we demonstrated the effect of a high

Figure 4 The effect of rosiglitazone treatment on blood glucose and lipid profiles in rats. LETO rats ($n=8$) were fed a normal chow diet and treated with a vehicle, while some OLETF rats ($n=9$) were fed a high-fat diet and treated with a vehicle, and the remaining OLETF rats ($n=8$) were fed a high-fat diet and treated with rosiglitazone (4 mg/kg per day) for 5 weeks. (A) An oral glucose tolerance test was performed in each experimental group. (B) The serum concentrations of total cholesterol and triglyceride in each experimental group. Data are mean $\pm$ S.E.M. * $P<0.05$ vs LETO rats; † $P<0.05$ vs OLETF rats.

Figure 5 The effect of diabetes and rosiglitazone treatment on hepatic LRP1 expression. (A) Western blot analysis of LRP1 (β -chain) in the liver samples of each experimental animal group. LETO rats ($n=8$) were fed a normal chow diet and treated with a vehicle, while some OLETF rats ($n=9$) were fed a high-fat diet and treated with a vehicle, and the remaining OLETF rats ($n=8$) were fed a high-fat diet and treated with rosiglitazone (4 mg/kg per day) for 5 weeks. (B) Western blot analysis of LRP1 (β -chain) in HepG2 cells ($n=6$ /group). HepG2 cells were incubated with indicated concentrations of glucose for 24 h and subsequently the cells were treated with indicated concentrations of rosiglitazone for 48 h. Results of western blot analysis were normalized to β -actin protein level and are expressed as ratios relative to LETO rats or to the nontreated cells in a normal glucose (5.5 mM) condition. Data are mean $\pm$ S.E.M. * $P<0.05$.

glucose condition on the expression of LRP1 in HepG2 cells. A high glucose condition (25 mM glucose in culture media) reduced the protein expression of LRP1 in HepG2 cells and rosiglitazone treatment recovered the LRP1 expression in a high glucose condition (Fig. 5B).

Discussion

Recently, Laatsch *et al.* (2009) reported that insulin induced the translocation of hepatic LRP1 from the cytosol to the plasma membrane and resulted in an increase in postprandial lipoprotein clearance. They showed that a glucose-induced insulin response increased the hepatic uptake of LRP1 ligands in WT mice while insulin-inducible LRP1 ligand uptake was abolished in leptin-deficient obese mice (*ob/ob*), which are characterized as being hepatic insulin resistant (Laatsch *et al.* 2009). The commentary on the study of Laatsch *et al.* indicated the clinical mechanistic importance of the study investigating the effects of insulin sensitizing drugs, such as thiazolidinedione, on the metabolism of hepatic LRP1 and remnant lipoproteins (Santos 2009). Before the study of Laatsch *et al.*, an *in vitro* study reported that rosiglitazone increased *LRP1* mRNA transcription and that the expression of LRP1 is regulated by PPAR γ in primary human adipocytes (Gauthier *et al.* 2003). They reported a conserved PPRE in the promoter region of the *LRP1* gene and showed that the upregulation of LRP1 by rosiglitazone is mediated by PPAR γ and a PPPE in the *LRP1* promoter at a transcriptional level in human adipocytes (Gauthier *et al.* 2003). If this PPAR γ -dependent regulatory mechanism of LRP1 was also effective in hepatocytes, we could suggest a novel mechanism of the beneficial effect of thiazolidinediones on the remnant lipoprotein clearance. Our data showed that rosiglitazone increased the expression of LRP1 in HepG2 cells and that 5-week treatment of rosiglitazone increased hepatic LRP1 expression in OLETF rats. This upregulation of LRP1 by rosiglitazone increased ApoE uptake in HepG2 cells. We also reaffirmed that the expression of LRP1 is regulated by PPAR γ in HepG2 cells. Our *in vitro* and *in vivo* data support our hypothesis suggesting that the hepatic uptake of remnant lipoproteins through LRP1 may contribute to the decrease in serum triglycerides and RLP-C levels due to thiazolidinedione treatment. Considering that this mechanism is not insulin dependent, increased hepatic LRP1 due to thiazolidinediones may contribute to the clearance of not only chylomicron remnants in a postprandial status but also VLDL remnants in a fasting status.

In this study, the increases in LRP1 mRNA transcription and protein expression were induced by

Figure 6 Immunohistochemistry of LRP1 in the liver samples of animal models. LETO rats ($n=8$) were fed a normal chow diet and treated with a vehicle, while some OLETF rats ($n=9$) were fed a high-fat diet and treated with a vehicle, and the remaining OLETF rats ($n=8$) were fed a high-fat diet and treated with rosiglitazone (4 mg/kg per day) for 5 weeks. Two samples of each group were presented and LRP1 expression was visualized by brown color.

100 nM rosiglitazone and were maintained by up to 5 μ M rosiglitazone in HepG2 cells. At concentrations above 50 μ M rosiglitazone, LRP1 expression did not change. This biphasic reaction to rosiglitazone was also seen for *LRP1* promoter activity. These results are consistent with a previous study using adipocytes. In primary human adipocytes, 50 nM rosiglitazone in culture media upregulated the transcription of LRP1 and the upregulation was maintained in up to 1 μ M rosiglitazone, but the induction of LRP1 expression by rosiglitazone diminished at a concentration of 2 μ M (Gauthier *et al.* 2003). And in SW872 cells, a human liposarcoma cell line, rosiglitazone concentration of 750 nM or higher did not alter the *LRP1* mRNA abundance or the LRP1 promoter activity (Gauthier *et al.* 2003). It was reported that the activation of PPAR γ at the AF2 domain by its ligands (PPAR γ agonists) increased its transcriptional function, and the same process enhanced subsequent proteosomal degradation of PPAR γ (Hauser *et al.* 2000). This would explain the reduced efficacy of rosiglitazone at higher concentrations (Gauthier *et al.* 2003). The pharmacokinetics of rosiglitazone in the human body reveals that the plasma concentration of rosiglitazone reaches the highest level – $\sim$ 300 ng/ml (840 nM) – after a single dose administration of 4 mg (Cox *et al.* 2000, Niemi *et al.* 2003). In this regard, the range of rosiglitazone concentration, which increased LRP1 in HepG2 cells (100 nM to 5 μ M), includes the serum concentration obtained with the conventional use of this drug.

Atherogenic dyslipidemia in DM patients includes high serum triglyceride and low serum HDL-C levels,

and this alteration in the lipid profile has been explained by an increase in hepatic VLDL synthesis resulting from insulin resistance (Gordon *et al.* 1989, Assmann & Schulte 1992, Bell *et al.* 2011). Our animal data showed that hepatic LRP1 was reduced in diabetic OLETF rats compared with levels in nondiabetic LETO rats. This result suggests that the alteration in hepatic clearance of triglyceride-rich remnant lipoproteins through LRP1 may be associated with the development of atherogenic dyslipidemia in a diabetic condition. The alteration of LRP1 in a diabetic status has been reported in one study investigating the blood–brain barrier (Hong *et al.* 2009), in which the expression of LRP1 in the brain microvessel was reduced in streptozotocin-induced diabetic mice (Hong *et al.* 2009). Because a streptozotocin-induced diabetic animal model is characterized by high serum glucose rather than insulin resistance and OLETF rats in our study also showed high serum glucose level, we investigated the effect of a high glucose condition on LRP1 expression in HepG2 cells. As expected, a high glucose condition decreased the expression of LRP1 in HepG2 cells. Our data suggest that hyperglycemia can be associated with a decrease in hepatic LRP1 and can alter the clearance of remnant lipoproteins from the circulation. Considering the alteration in the hepatic LRP1 translocation to the plasma membrane in an insulin-resistant animal model (Laatsch *et al.* 2009), the hepatic clearance of remnant lipoproteins through LRP1 in a diabetic condition might be reduced by a decrease in LRP1 itself as well as a decrease in the functional efficiency of LRP1. However, our data cannot exclude the possibility that factors associated with

insulin resistance altered the hepatic LRP1 expression. The mechanism of the decrease in hepatic LRP1 in a diabetic status should be elucidated in future studies.

One of the limitations of this study is the lack of serum RLP-C data. Currently available commercial kits are able to measure only human serum RLP-C levels. However, a strong correlation between serum RLP-C and serum triglyceride level has been reported (Imke *et al.* 2005). Another limitation of this study is that we did not demonstrate the direct association of altered hepatic LRP1 with serum triglyceride level *in vivo*. We observed an increase in serum triglyceride level along with decreased hepatic LRP1 in diabetic rats and a decrease in serum triglyceride level with increased hepatic LRP1 after rosiglitazone treatment. However, because other mechanisms that are associated with the change in serum triglyceride level have been illustrated in diabetes (Bell *et al.* 2011) and thiazolidinedione treatment (Hanefeld 2009), our *in vivo* data cannot conclude a causal relationship between hepatic LRP1 expression and serum triglyceride level. In addition, previous studies reported that hepatic LRP1 deficiency did not influence serum triglyceride level (Rohlmann *et al.* 1998, Basford *et al.* 2011). In this study, we used OLETF rats fed a high-fat diet as a diabetic animal model whereas previous studies used mice fed a normal chow diet. Differences in not only the animal model but also the diet could explain this discrepancy between our data and previous studies because plasma triglyceride level can be affected by the fat content of a diet. There is a possibility that the high-fat diet amplified the influence of hepatic LRP1 on serum triglyceride level. Despite of this limitation in our *in vivo* data, we demonstrated that ApoE uptake through LRP1 is induced by rosiglitazone in a human hepatoma-derived cell line, and these data suggest that a change in hepatic LRP1 can affect the clearance of triglyceride-rich remnant lipoproteins and serum triglyceride levels. Finally, we used rosiglitazone as a PPAR γ agonist, a thiazolidinedione class drug, in this study. Recently, rising concerns about the adverse effects of rosiglitazone have reduced the use of this agent, particularly since the association of rosiglitazone with an increased risk of adverse cardiovascular events has been reported. However, we demonstrated that rosiglitazone induced LRP1 expression in HepG2 cells via PPAR γ activation, and, therefore, we expect that other thiazolidinedione class drugs, such as pioglitazone, would have the same effect on LRP1 expression in hepatocytes. Our previous study using human brain microvascular endothelial cells showed that both rosiglitazone and pioglitazone increased LRP1 expression in endothelial cells (Moon *et al.* 2011).

In conclusion, rosiglitazone treatment increased LRP1 expression and function in HepG2 cells and in the livers of diabetic rats. In addition, a diabetic

condition including a high glucose condition decreased the expression of hepatic LRP1. These data suggest that decreased hepatic LRP1 in a diabetic condition is associated with the development of atherogenic dyslipidemia and that increased hepatic LRP1 by thiazolidinediones could contribute to an improvement in atherogenic lipid profiles in diabetic patients.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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