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Up-regulation of hepatic low-density lipoprotein receptor–related protein 1: a possible novel mechanism of antiatherogenic activity of hydroxymethylglutaryl–coenzyme A reductase inhibitor Atorvastatin and hepatic LRP1 expression

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Abstract

Low-density lipoprotein receptor–related protein 1 (LRP1) binds to apolipoprotein E and serves as a receptor for remnant lipoproteins in the liver, thus playing an important role in clearing these atherogenic particles. In this study, we investigated the effect of atorvastatin, a hydroxymethylglutaryl–coenzyme A reductase inhibitor, on hepatic LRP1 expression. We used HepG2 and Hep3B cells for in vitro study, and Otsuka Long-Evans Tokushima fatty and Sprague-Dawley rats for in vivo study. We used relatively high pharmacologic dose of atorvastatin in this study (in vitro, 0.5 μmol/L in culture media, for 48 hours; in vivo, 20 mg/[kg d], for 6 weeks). Atorvastatin increased LRP1 and low-density lipoprotein (LDL) receptor expression in HepG2 and Hep3B cells and induced hepatic LRP1 and LDL receptor expression in chow diet-fed Sprague-Dawley rats and high-fat diet–fed Otsuka Long-Evans Tokushima fatty rats. Atorvastatin decreased intracellular sterol level and increased the amount of the nuclear form of sterol response element-binding protein–2 (SREBP-2) in both HepG2 and Hep3B cells as well as in two animal models. Treatment of HepG2 cells with LDL increased intracellular sterol level and reduced LRP1, LDL receptor, and SREBP-2. When SREBP-2 in HepG2 cells was knocked down by small interfering RNA, the induction of LRP1 expression by atorvastatin did not take place. In conclusion, up-regulation of hepatic LRP1 might be a novel mechanism by which statin treatment decreases remnant lipoproteins. In addition, SREBP-2 acts as a mediator of atorvastatin-induced up-regulation of hepatic LRP1. Future studies using standard doses of atorvastatin in humans are needed to elucidate clinical relevance of these findings.

1. Introduction

Low-density lipoprotein receptor–related protein 1 (LRP1), a member of the low-density lipoprotein (LDL) receptor gene family, is a multifunctional scavenger and signaling receptor [1]. This cell surface glycoprotein binds to apolipoprotein E and serves as a receptor for chylomicron remnants in the liver [2]. Furthermore, LRP1 plays an important role in the clearance of remnant of triglyceride-rich lipoproteins such as chylomicron remnant or very low-density lipoprotein remnant [3,4]. Recent research has focused on these remnant lipoproteins as atherogenic particles and an independent risk factor of cardiovascular disease or stroke [5-7]. Remnant lipoproteins have been

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reported to be associated with inflammation, endothelial dysfunction, formation of foam cells, and proliferation of vascular smooth muscle cells (VSMCs) [8-11]. In addition, type 2 diabetes mellitus (T2DM) patients have high levels of remnant lipoproteins [12-14].

Hydroxymethylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors, or "statins," inhibit cellular cholesterol biosynthesis and result in increased activity of the LDL receptors and enhanced clearance of lipoproteins containing apolipoprotein B [15,16]. Early use of statins is recommended by current guidelines for cardiovascular risk prevention in T2DM [17]. These agents have also been reported to reduce remnant-like particle cholesterol (RLP-C) in patients with T2DM or hypercholesterolemia [18-24]. Considering the adverse effects of remnant lipoproteins on cardiovascular disease especially in diabetic status, understanding the effect of widely used antihyperlipidemic drugs on hepatic LRP1 expression and the regulatory mechanism of this scavenger receptor in the liver might provide important clues to help lower the risk of cardiovascular disease. In this study, we investigated the effect of atorvastatin, one of the HMG-CoA reductase inhibitors, on hepatic LRP1 expression in animal models and also investigated a possible mechanism of hepatic LRP1 expression involving the regulation of sterol regulatory element binding protein–2 (SREBP-2) in response to intracellular cholesterol level. This is the first study to investigate the regulation of LRP1 expression in the liver and hepatocyte-derived cell line.

2. Materials and methods

2.1. Cell culture

The HepG2 cells and Hep3B cells were cultured in modified Eagle medium containing 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 mg/mL) at 37°C in 5% CO_2/95% air. Atorvastatin (200 mmol/L; Yuhan, Corp, Seoul, Korea) was prepared in ethanol at −20 °C. A stock solution of LDL (Sigma-Aldrich, St Louis, MO) was prepared in tertiary distilled water at 5 mg/mL and stored at −4 °C. HepG2 cells and Hep3B cells were treated with different concentrations of atorvastatin (0, 0.1, 0.5, 1, 10, and 50 μmol/L in culture media) or LDL (0, 40, and 200 μg/mL in culture media) for 48 hours by adding the stock solution directly to the culture media. The final concentration of ethanol in the culture media was adjusted to 0.2% (vol/vol).

2.2. Small interfering RNA transfections

Small interfering RNA (siRNA) targeting SREBP-2 (siSREBP-2) and scrambled RNA (siCTRL) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The sequence information is protected by the provider. Each siRNA was transfected into HepG2 cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA). After an overnight transfection, the cells were treated with atorvastatin (0.5 μmol/L in culture media) for 48 hours, respectively. We determined siRNA silencing efficiency by a quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) of SREBP-2 messenger RNA (mRNA) in siSREBP-2- and siCTRL-transfected HepG2 cells.

2.3. Animals, diet, and treatment

Twenty-week-old Otsuka Long-Evans Tokushima fatty (OLETF) rats (Otsuka Pharmaceutical, Tokushima, Japan) and Sprague-Dawley (SD) rats (Central Laboratory Animal, Seoul, Korea) were maintained at ambient temperature (22°C ± 1°C) on 12-hour light-dark cycles with free access to water and diet. The OLETF rats were fed a high-fat diet containing 40% lard oil (Wellga, Seoul, Korea) and were randomly divided into 2 groups at 20 weeks of age. The rats in the control group (n = 10) were untreated and killed at 26 weeks of age; the rats in the atorvastatin group (n = 10) were treated with atorvastatin (Lipitor, Pfizer Korea; 20 mg/[kg d] dissolved in 1 mL of drinking water) by oral administration with a 10-mL syringe for 6 weeks from 20 weeks of age and were killed at 26 weeks of age. The SD rats were fed a normal chow diet and were randomly divided into 2 groups in the same manner as OLETF rats at 9 weeks of age. The SD rats were killed at 15 weeks of age. The animals were euthanized at the end of a dark cycle after overnight fasting for tissue sampling. Blood was collected by cardiac puncture; and the livers were isolated, immediately freeze-clamped in liquid nitrogen, and stored at −80 °C until analysis. All procedures were approved by the Institutional Animal Care and Use Committee at the Yonsei University College of Medicine.

2.4. Oral glucose tolerance test

An oral glucose tolerance test (OGTT) was performed in OLETF rats after administering 20% glucose solution (2 g/kg) followed by an overnight fast. Blood samples were obtained by tail snipping at the indicated time, and blood glucose levels were measured with a glucose analyzer (Accu-Check; Roche Diagnostics, Basel, Switzerland).

2.5. Immunofluorescent staining analysis

Rat livers were fixed in 10% formaldehyde and embedded in paraffin. The paraffin sections were cut and deparaffinized using xylene and ethanol. After a 30-minute incubation in 5% bovine serum albumin for blocking, samples were incubated with anti-LRP1 antibody (rabbit monoclonal immunoglobulin [Ig] G, Santa Cruz Biotechnology) for 1 hour at room temperature and then overnight at 4°C. After the primary antibody reaction, samples were incubated with fluorescein isothiocyanate–tagged goat anti-rabbit IgG (Santa Cruz Biotechnology) for 40 minutes at room temperature and were mounted with antifading universal mounting solution (Santa Cruz Biotechnology).

2.6. RNA and complementary DNA preparation

Total RNA was isolated from rat liver tissue and hepatocyte-derived cells using Trizol reagent (Invitrogen) and quantified by nanodrop (ND-1000; DM Science, Seoul, Korea). Following RNA extraction, 4 μL RNA was treated with 1 unit DNase I to remove all contaminating genomic DNA. DNase-treated RNA was subsequently used for complementary DNA (cDNA) synthesis using MMLV reverse transcriptase (Promega, Madison, WI): 1 μL oligo dT primer was added to 4 μL RNA with 5× MMLV reaction buffer, 2.5 mmol/L each dNTP, 1 unit RNasin ribonuclease inhibitor, and MMLV reverse transcriptase (200 units). Complementary DNA was stored at −20 °C.

2.7. Quantitative RT-PCR

Real-time quantitative RT-PCR analysis of various genes was performed with an ABI 7500 instrument and software (Applied Biosystems, Foster City, CA). All Taqman assays were performed with inventoried primers and probes (Applied Biosystems): β-actin (Hs99999903_m1, R700667669_m1), SREBP-2 (Hs01081778_m1, R701502638_m1), LRP1 (Hs00233856_m1, R701503964 g1), and LDL receptor (Hs00181192_m1, Rn00598438). The PCRs were carried out in triplicate in a final volume of 20 μL according to the manufacturer's protocol. For each assay, a standard curve was obtained by analyzing a dilution series of pooled cDNA samples for the relevant gene. Data were analyzed with Sequence Detector 1.7 software (Applied Biosystems) β-Actin was used as the internal standard to control for variability, and the results were expressed as a ratio of the gene expression relative to that of β-actin.

2.8. Immunoblot analysis

Aliquots of cell lysates (Thermo Scientific, Waltham, MA) and tissue homogenates were denatured under reducing conditions (1.75% sodium dodecyl sulfate, 15 mmol/L 2-mercaptoethanol; 5 minutes at 100°C), then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblot analyses. To detect LRP1, nitrocellulose membranes were incubated with anti-LRP1 antibody (rabbit monoclonal IgG; Epitomics, Burlingame, CA) (1:1500, overnight at 4°C) and then with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000, Santa Cruz Biotechnology) for 1 hour. Immunolabeling was detected with the ECL Western Blotting Analysis System (Thermo Scientific). The LRP immunoreactivity was normalized to total protein as determined by Bradford assay (Sigma-Aldrich). The LDL receptor was detected with anti-LDL receptor antibody (rabbit polyclonal IgG, 1:1000; Abcam, Cambridge, United Kingdom) and a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (1:5000, Santa Cruz Biotechnology). The nuclear form of SREBP-2 was detected with anti-SREBP-2 antibody (rabbit polyclonal IgG, 1:1000, Santa Cruz Biotechnology) and a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (1:5000, Santa Cruz Biotechnology). β-Actin immunoreactivity, detected with monoclonal anti–β-actin antibody (1:5000, Sigma-Aldrich) and a goat anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (1:5000, Santa Cruz Biotechnology), was used as a loading control.

2.9. Intracellular sterol assay

Intracellular sterol was extracted in 200 mg of liver tissue or hepatocyte-derived cells. Total cholesterol was determined by enzymatic methods (Roche Diagnostics).

2.10. Statistical analysis

All statistical analyses were performed with SPSS software (version 15.0; SPSS, Chicago, IL). Values were expressed as the mean ± SD. Statistical comparison between groups was performed using the Student *t* test or 1-way analysis of variance. Data with a P value < .05 were considered significant.

3. Results

3.1. Atorvastatin treatment increased LRP1 and LDL receptor expression in HepG2 and Hep3B cells

To find out the optimal dose of atorvastatin, we treated increasing concentrations of atorvastatin on HepG2 cells. Although we detected up-regulation of LRP1 expression in 0.1 μmol/L of atorvastatin (Figure S1), we treated 0.5 and 10 μmol/L of atorvastatin for clear data. HepG2 cells are widely used in the studies to investigate the lipid metabolism in hepatocyte, and we also used Hep3B cells to obtain both exact and generalized results. Atorvastatin treatment increased LRP1 mRNA and protein expression in HepG2 cells (control vs 0.5 μmol/L atorvastatin, mRNA, 1.00 ± 0.16 vs 2.47 ± 0.34 arbitrary units [AU], P = .004; protein, 1.00 ± 0.26 vs 2.27 ± 0.33 AU, P = .013) and Hep3B cells (control vs 0.5 μmol/L atorvastatin, mRNA, 1.00 ± 0.08 vs 1.52 ± 0.21 AU, P = .025; protein, 1.00 ± 0.18 vs 1.65 ± 0.19 AU, P = .018) (Fig. 1). Atorvastatin induced LDL receptor expression in HepG2 cells (control vs 0.5 μmol/L atorvastatin, mRNA, P = .003; protein, P = .006) and Hep3B cells (control vs 0.5 μmol/L atorvastatin, mRNA, P = .01; protein, P = .008) (Fig. 1).

3.2. Atorvastatin treatment increased hepatic LRP1 and LDL receptor expression in SD rats as well as OLETF rats

We administrated atorvastatin (20 mg/[kg d], for 6 weeks) to chow diet-fed SD rats and high-fat diet–fed OLETF rats. We used OLETF rats as the T2DM animal model and used SD rats to investigate the effect of atorvastatin on the normal metabolic status. Atorvastatin increased hepatic LRP1 expression in OLETF rats (control vs atorvastatin, mRNA 1.00 ± 0.23 vs 2.91 ± 0.42 AU, P = .001; protein, mRNA 1.00 ± 0.11 vs 1.98 ± 0.18 AU, P = .001) and SD rats (control vs atorvastatin, mRNA 1.00 ± 0.11 vs 1.54 ± 0.10 AU, P = .003; protein, 1.00 ± 0.09 vs 1.58 ± 0.12 AU, P = .001) (Fig. 2).

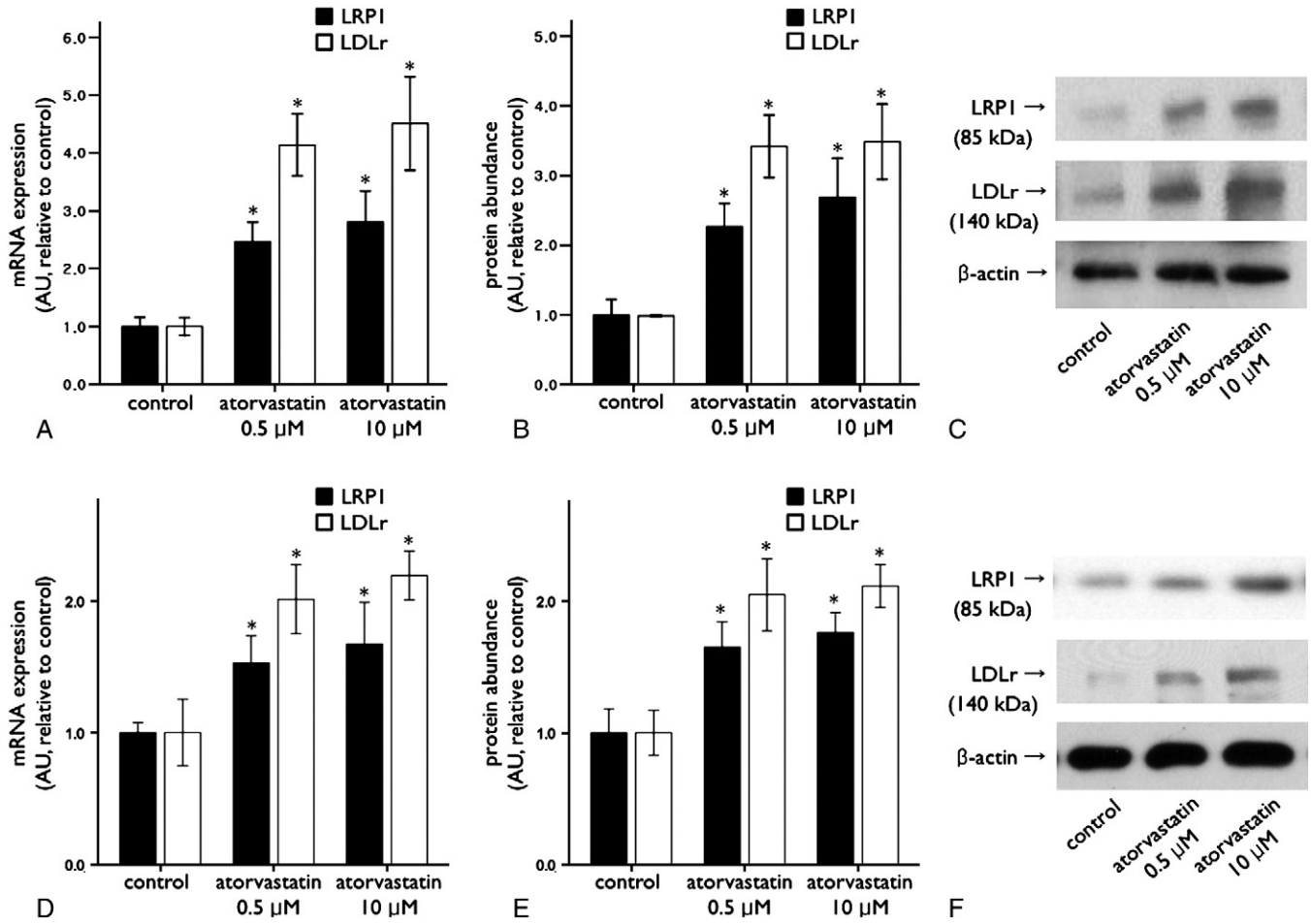

Fig. 1. Effect of atorvastatin on LRP1 and LDL receptor expression in hepatocyte-derived cell lines. HepG2 and Hep3B cells were incubated with different concentrations of atorvastatin (0, 0.5, and 10 μmol/L in culture media) for 48 hours. A, Real-time PCR quantification of LRP1 and LDL receptor mRNA in HepG2 cells. B and C, Western blot analysis of LRP1 (β-chain) and LDL receptor in HepG2 cells. D, Real-time PCR quantification of LRP1 and LDL receptor mRNA in Hep3B cells. E and F, Western blot analysis of LRP1 (β-chain) and LDL receptor in Hep3B cells. Results were normalized by β-actin mRNA or protein level and are expressed as ratio relative to controls. Data are mean ± SD. *$P < .05$ vs control. LDLr indicates low-density lipoprotein receptor.

LDL receptor was also induced by atorvastatin treatment in OLETF rats (mRNA, $P < 6$ .001; protein, $P = .001$) and SD rats (mRNA, $P = .015$; protein, $P = .006$) (Fig. 2). Immunofluorescent staining of OLETF rat liver also showed increased LRP1 expression following atorvastatin treatment (Fig. 3).

3.3. Atorvastatin reduced intracellular cholesterol and increased the nuclear form of SREBP-2 in hepatocyte-derived cell lines and rat liver

We evaluated the effect of atorvastatin on intracellular cholesterol level and the expression of the nuclear form of SREBP-2. In HepG2 cells, atorvastatin reduced intracellular cholesterol level (control vs 0.5 μmol/L atorvastatin, 100.0 ± 2.9 vs 87.1 ± 7.6 AU, $P = .015$) and increased mRNA expression and protein abundance of the nuclear form of SREBP-2 (control vs 0.5 μmol/L atorvastatin, mRNA, $P < .001$; protein, $P = .001$) (Fig. 4A-D). Atorvastatin also decreased intracellular cholesterol level (control vs 0.5 μmol/L atorvastatin, 100.0 ± 4.6 vs 87.0 ± 7.5 AU, $P = .011$) and increased the expression of the nuclear form of SREBP-2 in Hep3B cells (control vs 0.5 μmol/L atorvastatin, mRNA, $P < .001$; protein, $P = .001$) (Fig. 4E-H).

In the livers of OLETF rats, the hepatic cholesterol level was reduced by atorvastatin treatment (control vs atorvastatin, 100.0 ± 10.0 vs 63.4 ± 6.0 AU, $P = .011$) (Fig. 5A). As expected, atorvastatin treatment increased SREBP-2 mRNA expression ($P = .003$) as well as the level of the nuclear form of SREBP-2 ($P = .001$) (Fig. 5B-D). Atorvastatin treatment in SD rats showed the same result as in OLETF rats (control vs atorvastatin, cholesterol, 100.0 ± 9.6 vs 74.8 ± 6.7 AU, $P = .001$; SREBP-2, mRNA, $P < .001$; protein, $P = .008$) (Fig. 5E-H).

3.4. LDL increased intracellular cholesterol level and reduced SREBP-2, LRP1, and LDL receptor expressions in HepG2 cells

To test the effect of increased intracellular cholesterol level on LRP1 expression in HepG2 cells, we treated HepG2

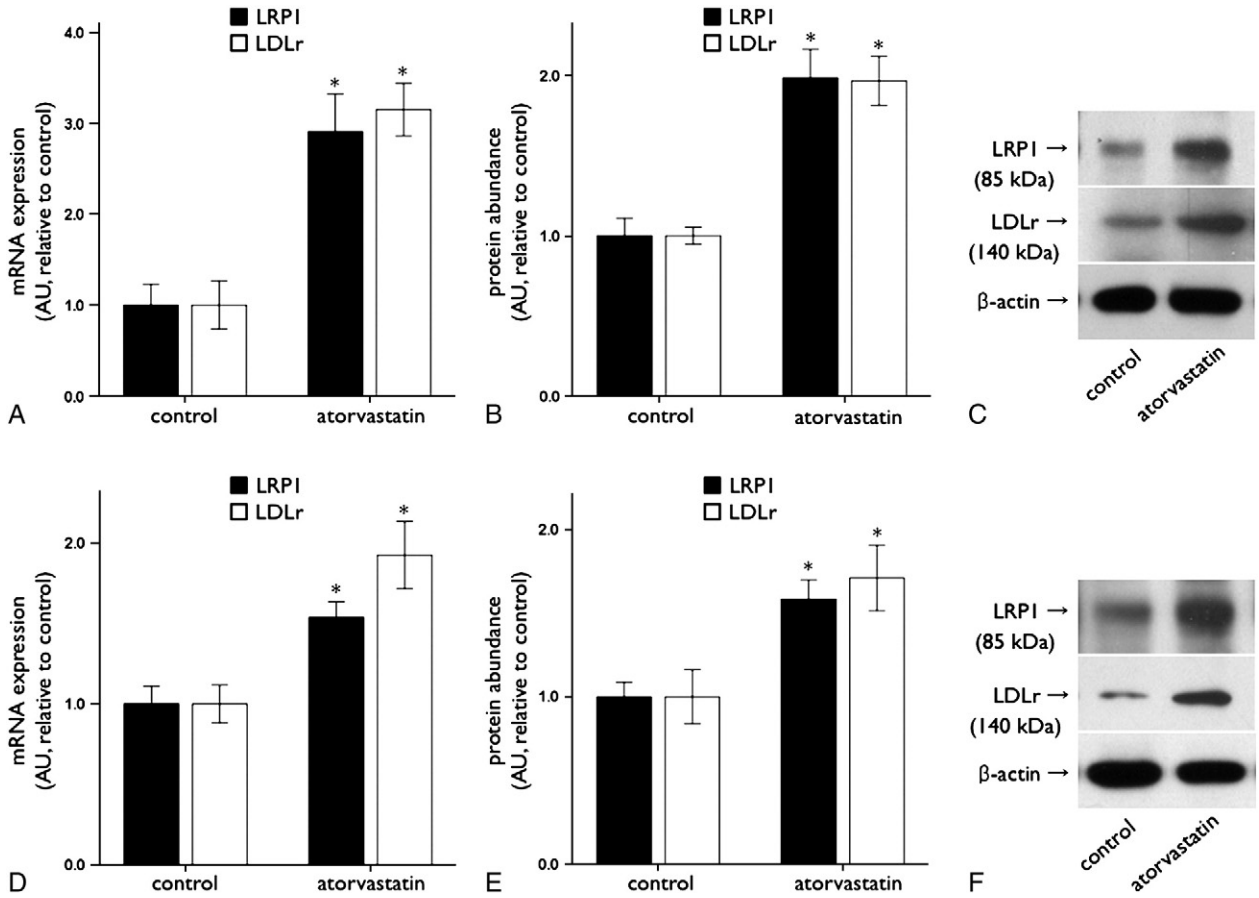

Fig. 2. Effect of atorvastatin on LRP1 and LDL receptor expression in the livers of OLETF and SD rats. Rats were fed with or without atorvastatin (20 mg/[kg d] for 6 weeks) in drinking water. A, Real-time PCR quantification of LRP1 and LDL receptor mRNA in liver samples of OLETF rats. B and C, Western blot analysis of LRP1 (β-chain) and LDL receptor in liver samples of OLETF rats. D, Real-time PCR quantification of LRP1 and LDL receptor mRNA in liver samples of SD rats. E and F, Western blot analysis of LRP1 (β-chain) and LDL receptor in liver samples of SD rats. Results were normalized by β-actin mRNA or protein level and are expressed as ratio relative to controls. Data are mean ± SD. *$P < .05$ vs control.

cells with different concentrations of LDL (40 and 200 μg/mL in culture media). High-dose LDL treatment increased intracellular cholesterol level in HepG2 cells (control vs 200 μg/mL LDL, 100.0 ± 2.7 vs 119.3 ± 3.8 AU, $P = .038$) (Fig. 6A). The SREBP-2 mRNA expression was not affected by treatment with 40 μg/mL LDL, but 200 μg/mL LDL reduced SREBP-2 mRNA expression ($P < .001$) and the level of the nuclear form of SREBP-2 protein ($P < .001$) (Fig. 6B, C and F). Interestingly, mRNA expression and protein abundance of LRP1 and LDL receptor were affected in a similar manner. Low-density lipoprotein treatment decreased LDL receptor and LRP1 expression in HepG2 cells (control

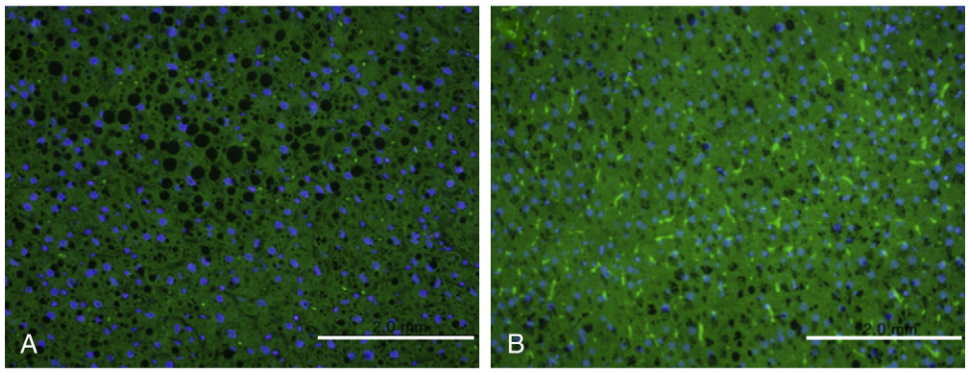

Fig. 3. Immunofluorescence staining using LRP1 (α chain)-specific antibody in OLETF rat. A, The liver of an OLETF rat without atorvastatin treatment. B, The Liver of an OLETF rat with atorvastatin treatment (20 mg/[kg d], for 6 weeks). The LRP1 (α chain) showed light green color in the picture. Fluorescein isothiocyanate–tagged secondary antibody was used for detection of LRP1. Bars represent 2.0 mm.

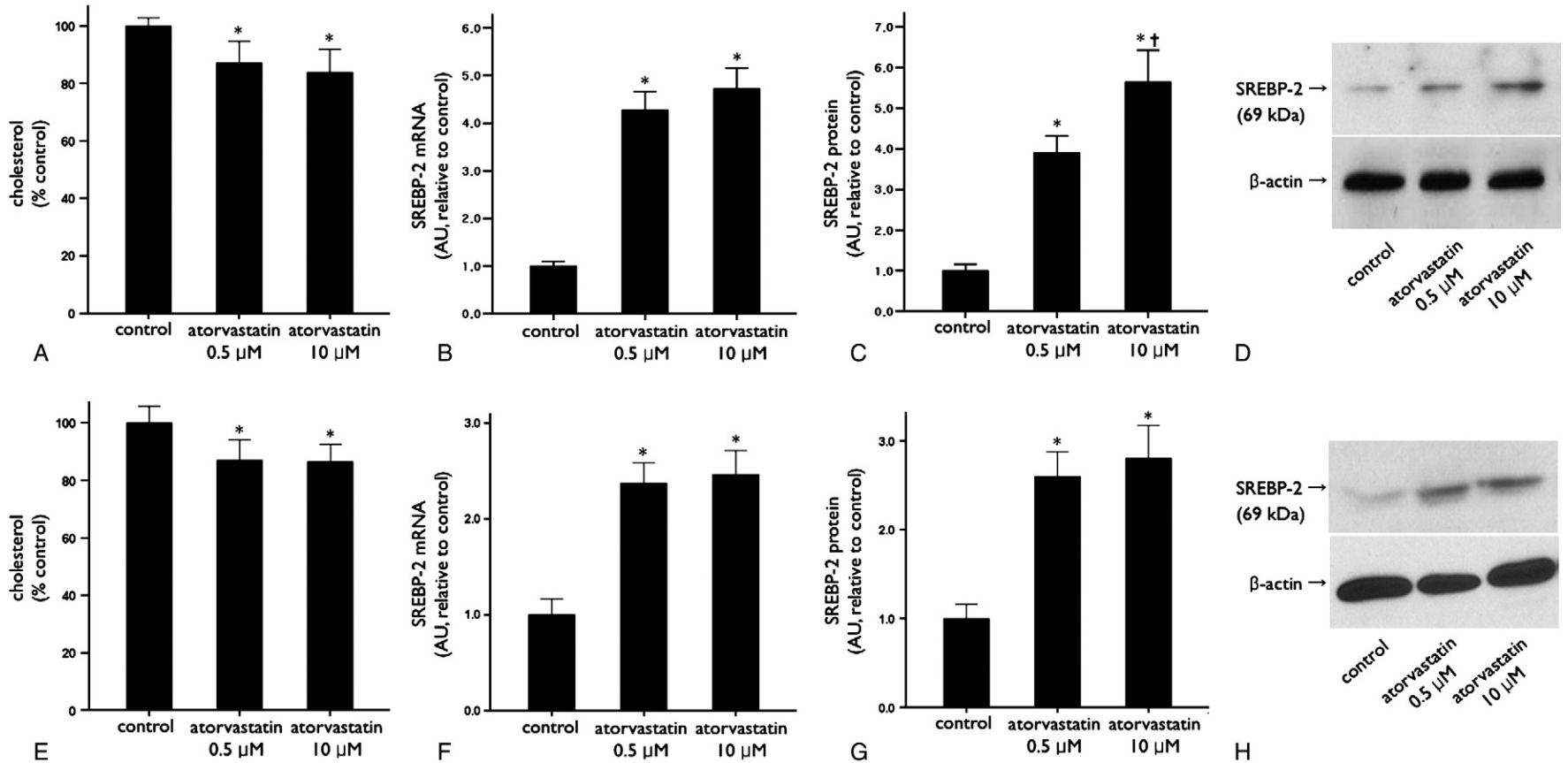

Fig. 4. Effects of atorvastatin on intracellular cholesterol level and SREBP-2 expression in hepatocyte-derived cell lines. HepG2 and Hep3B cells were incubated with different concentrations of atorvastatin (0, 0.5, and 10 μmol/L in culture media) for 48 hours. A, Enzyme-linked immunosorbent assay quantification of intracellular cholesterol in HepG2 cells. Cholesterol level was normalized by cellular protein. B, Real-time PCR quantification of SREBP-2 mRNA in HepG2 cells. C and D, Western blot analysis of SREBP-2 in HepG2 cells. E, Enzyme-linked immunosorbent assay quantification of intracellular cholesterol in HepG2 cells. Cholesterol level was normalized by cellular protein. F, Real-time PCR quantification of SREBP-2 mRNA in HepG2 cells. G and H, Western blot analysis of SREBP-2 in HepG2 cells. Results were normalized by β-actin mRNA or protein level and are expressed as ratio relative to controls. Data are mean ± SD. *$P < .05$ vs control; $^{\dagger}P < .05$ vs atorvastatin 0.5 μmol/L.

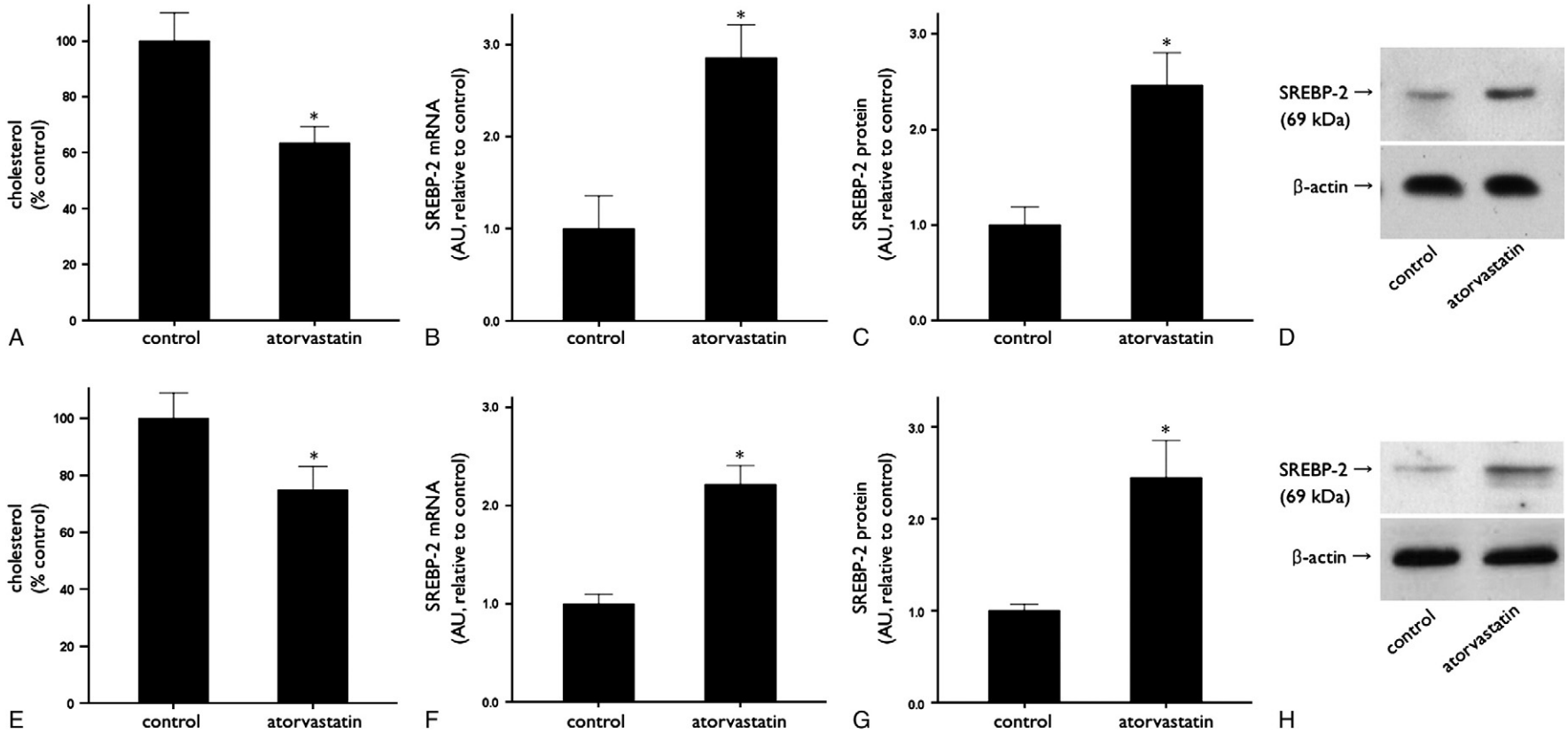

Fig. 5. Effects of atorvastatin on intracellular cholesterol level and SREBP-2 expression in the livers of OLETF and SD rats. The OLETF rats were fed with or without atorvastatin (20 mg/[kg d] for 6 weeks) in drinking water. A, Enzyme-linked immunosorbent assay quantification of cholesterol in liver samples of OLETF rats. Cholesterol level was normalized by cellular protein. B, Real-time PCR quantification of SREBP-2 mRNA in liver samples of OLETF rats. C and D, Western blot analysis of SREBP-2 in liver samples of OLETF rats. E, Enzyme-linked immunosorbent assay quantification of cholesterol in liver samples of SD rats. Cholesterol level was normalized by cellular protein. F, Real-time PCR quantification of SREBP-2 mRNA in liver samples of SD rats. G and H, Western blot analysis of SREBP-2 in liver samples of SD rats. Results were normalized by β-actin mRNA or protein level and are expressed as ratio relative to controls. Data are mean ± SD. *$P < .05$ vs control.

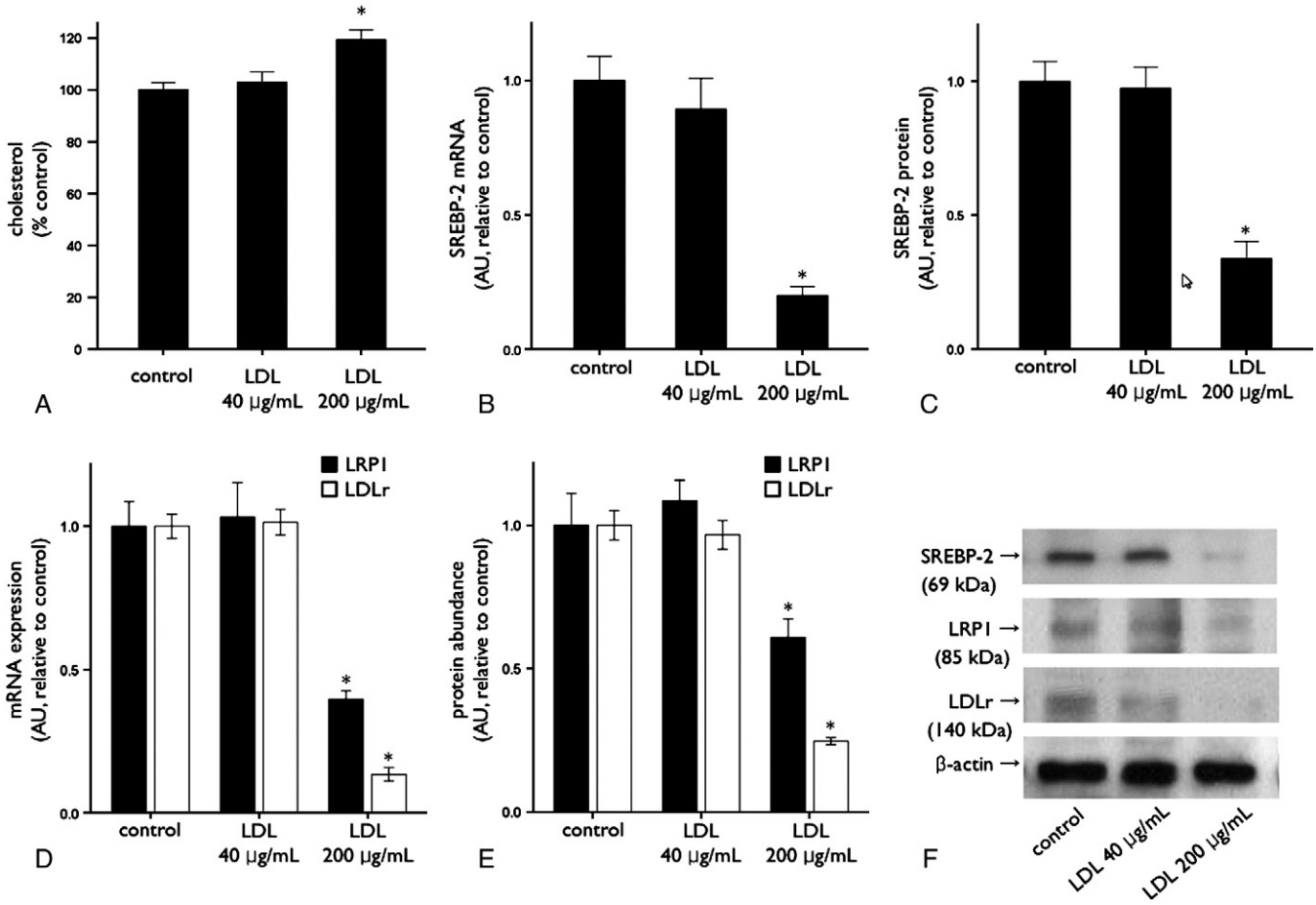

Fig. 6. Effect of LDL treatment on intracellular cholesterol level and the expressions of SREBP-2, LRP1, and LDL receptor in HepG2 cells. HepG2 cells were incubated with different concentrations of LDL (0, 40, and 200 μg/mL in culture media) for 48 hours. A, Enzyme-linked immunosorbent assay quantification of intracellular cholesterol. Cholesterol level was normalized by cellular protein. B, Real-time PCR quantification of SREBP-2 mRNA. C and F, Western blot analysis of SREBP-2. D, Real-time PCR quantification of LRP1 and LDL receptor mRNA. E and F, Western blot analysis of LRP1 and LDL receptor. Results were normalized by β-actin mRNA or protein level and are expressed as ratio relative to controls. Data are mean ± SD. *$P < .05$ vs control.

vs 200 μg/mL LDL, LDL receptor, mRNA, $P < .001$; protein, $P < .001$; LRP1, mRNA, 1.00 ± 0.09 vs 0.40 ± 0.03 AU, $P = .001$; protein, 1.00 ± 0.11 vs 0.61 ± 0.06 AU, $P = .011$) (Fig. 6D-F).

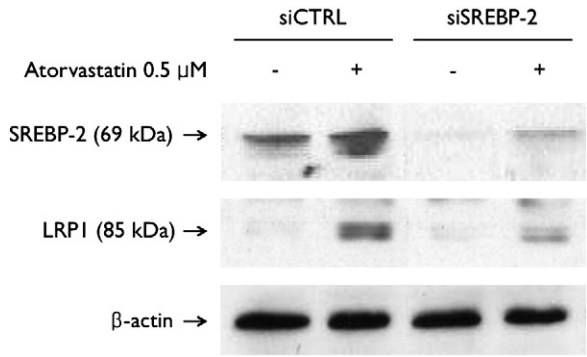

Fig. 7. Effect of SREBP-2 knockdown by siRNA on LRP1 expression in HepG2 cells treated with atorvastatin. HepG2 cells were transfected with scrambled RNA or siRNA targeting SREBP-2 and treated with atorvastatin (0.5 μmol/L in culture media). Western blot analyses of SREBP-2 and LRP1 were performed.

3.5. siRNA targeting SREBP-2 prevents atorvastatin-induced LRP1 up-regulation in HepG2 cells

We transfected HepG2 cells with siCTRL and siSREBP-2 to investigate the role of SREBP-2 in LRP1 regulation. The gene silencing effect of siSREBP-2 is shown in Fig. 7, and the transfection efficiency determined by real-time RT-PCR of SREBP-2 was 74.8% ± 8.3%. HepG2 cells transfected with siCTRL showed increased LRP1 expression following atorvastatin treatment. However, atorvastatin had a much smaller effect on the expression of LRP1 in HepG2 cells transfected with siSREBP-2 (Fig. 7).

4. Discussion

In HepG2 cells, atorvastatin decreases intracellular cholesterol and increases levels of SREBP-2 protein [25]. In our study, atorvastatin reduced the intracellular cholesterol level and up-regulated the nuclear form of SREBP-2 in HepG2 and Hep3B cells. In addition, LRP1 was up-regulated by atorvastatin treatment in these hepatocyte-

derived cell lines. We confirmed our results in vivo using 2 animal models. We administered atorvastatin for 6 weeks to OLETF rats and SD rats. The OLETF rats were diagnosed with diabetes by OGTT (Figure S2). The hepatic intracellular cholesterol level was reduced, and the nuclear form of SREBP-2 was increased by atorvastatin in both animal models. Atorvastatin treatment also increased hepatic LRP1 expression in the rats. These results suggest that induction of LRP1 by atorvastatin is observed not only in specific cell lines but also in the liver of an animal model. Some studies reported that HMG-CoA reductase inhibitor has no significant effect on hepatic LRP1 expression [26,27]. In those studies, rosuvastatin (20 mg/kg for 2 weeks) or lovastatin (0.2% [wt/wt] in diet for 1 week) was administered to SD rats or C57BL/6 mice, respectively. In our study, we maintained atorvastatin treatment for 6 weeks. Because there is no difference in daily dose of medication, there is a possibility that the difference in treatment duration made this discrepancy and that long-term treatment of HMG-CoA reductase inhibitor is required to have an effect on hepatic LRP1 expression in vivo. We also measured LDL receptor expression in the cell lines and animal models as a kind of positive control because hepatic LDL receptor is known to be induced by HMG-CoA reductase inhibitors via SREBP-2 [27]. As expected, LDL receptor expression was increased by atorvastatin both in hepatocyte-derived cell lines and in the livers of animal models. Altogether, hepatic LRP1 and LDL receptor expression showed similar response to atorvastatin treatment. Although we did not measure the level of RLP-C in rat blood, HMG-CoA reductase inhibitors including atorvastatin have been shown to reduce plasma RLP-C in several clinical studies [18-24]. The known mechanism of remnant lipoprotein reduction by HMG-CoA reductase inhibitors involves up-regulation of the hepatic LDL receptor via SREBP-2 and decreased hepatic secretion of apolipoprotein B–containing lipoproteins. In this study, we suggest a possible novel mechanism through which atorvastatin reduces plasma remnant lipoproteins; in this model, hepatic LRP1 is also up-regulated by atorvastatin and may function to clear remnant lipoproteins. Considering that the hepatic LDL receptors and LRP1 are both major contributors to the clearance of remnant lipoproteins (accounting for approximately 80% of the clearance in mice) [28], up-regulation of LRP1 and the LDL receptor by atorvastatin might explain almost all of the reduction in remnant lipoproteins.

To elucidate the mechanism through which atorvastatin induces hepatic LRP1 expression, we focused on the change in intracellular cholesterol level and the induction of SREBP-2 by atorvastatin treatment. We investigated LRP1 expression in HepG2 cells treated with LDL to evaluate the expression of LRP1 under conditions of increased intracellular cholesterol levels. As expected, LDL treatment of HepG2 cells increased the intracellular cholesterol level and decreased the expression of the nuclear form of SREBP-2. In addition, LRP1 mRNA and protein expressions were decreased by LDL treatment. These results suggest that hepatic LRP1 expression is negatively associated with intracellular cholesterol level and positively associated with expression of SREBP-2, as shown for LDL receptors. However, our results are opposite to those of studies investigating the regulation of LRP1 expression in other cell types. The LDL receptor has a sterol regulatory element (SRE) in its promoter region; therefore, high intracellular cholesterol reduces transcription of the LDL receptor gene via down-regulation of SREBP-2 [27]. However, a previous study showed that the promoter region of *LRP1* contains no SRE and that the expression of LRP1 is not regulated by the intracellular cholesterol level in mouse fibroblast and macrophage-derived cell lines [29]. Subsequently, the SRE site was found in an unusually long 5′–untranslated region (UTR) of *LRP1*, rather than in its promoter region [30]. Llorente-Cortés et al [31-33] have investigated the regulation of LRP1 in VSMCs and macrophages. They revealed that high LDL concentration and cellular accumulation of cholesteryl ester increases LRP1 expression in VSMCs and indicated a possible involvement of SREBP-2 [31]. In addition, they showed that SREBP-2 bound to the SRE sequence located in the 5′-UTR of *LRP1* [32] and negatively regulated LRP1 expression in human macrophages [33]. In our study, however, LRP1 expression in HepG2 cells was negatively associated with intracellular cholesterol and positively associated with SREBP-2 expression. These results suggest a possibility that, at least in hepatocytes, unlike extrahepatic tissues, LRP1 is regulated by intracellular cholesterol level via SREBP-2 and that SREBP2 acts as a key activator of *LRP1* transcription.

Although we showed that LRP1 expression is positively associated with SREBP-2, our results do not mean that SREBP-2 directly up-regulates LRP1 expression. To examine the role of SREBP-2 in hepatic LRP1 regulation, we treated atorvastatin on HepG2 cells transfected with siRNA targeting SREBP-2. Expression of SREBP-2 and LRP1 was increased by atorvastatin in HepG2 cells transfected with siCTRL, but LRP1 up-regulation by atorvastatin was weak in HepG2 cells transfected with siSREBP-2. These results support our hypothesis that SREBP-2 is an activator of *LRP1* transcription in hepatocyte. Although it is clear that SREBP-2 affects the regulation of hepatic LRP1 expression, we cannot fully explain the mechanism of LRP1 up-regulation by atorvastatin because SREBP-2 gene silencing did not completely prevent the induction of LRP1 by atorvastatin. Although we did not examine whether SREBP-2 binds to the SRE sequence in 5′-UTR of *LRP1* in HepG2 cells, we did demonstrate that SREBP-2 plays a pivotal role in inducing LRP1 expression in HepG2 cells by silencing the SREBP-2 gene with siRNA. The reason for this discrepancy in LRP1 regulation according to cell type is unclear, but our results suggest that the action of SREBP-2 on the *LRP1* gene in hepatocytes differs from that in extrahepatic tissue such as VSMCs or macrophages.

Further investigation is needed to understand the differences in LRP1 regulation between the liver and

extrahepatic tissue. Investigating whether SREBP-2 binds to the SRE sequence in the 5′-UTR of the LRP1 gene in hepatocyte-derived cell lines and the effects of atorvastatin on LRP1 expression in extrahepatic tissue such as VSMCs, macrophages, or adipocytes would be interesting subjects. Determining the binding site of SREBP-2 in the LRP1 gene responsible for activating transcription and identifying any cofactors or liver-enriched factors required for SREBP-2–induced *LRP1* transcription might help us understand this discrepancy. In addition, considering the effect of SREBP-2 on LRP1 expression in extrahepatic tissue, HMG-CoA reductase inhibitors are expected to reduce LRP1 expression by up-regulating SREBP-2 in VSMCs or macrophages. Interestingly, in macrophages or VSMCs, LRP1 has been reported to be a key receptor for intracellular cholesterol accumulation and progression of atherosclerosis [31,33,34]. However, in the liver, LRP1 is one of the major receptors that clear atherogenic particles, together with the LDL receptor [28]. In light of this, our results suggest that HMG-CoA reductase inhibitors might have "dual" antiatherogenic effects by increasing the expression of LRP1 and the LDL receptor in the liver and decreasing the expression of LRP1 in VSMCs or macrophages.

This study also includes some important clinical issues. Recently, Laatsch et al [35] reported that insulin stimulated the translocation of hepatic LRP1 to the plasma membrane and increased hepatic uptake of LRP1-specific ligands. They also demonstrated insulin-inducible LRP1 ligand uptake was abolished in leptin-deficient obese mice (*ob*/*ob*) [35]. Our results in a diabetic animal model suggest that HMG-CoA reductase inhibitors might overcome this decreased response of hepatic LRP1 in insulin-resistant conditions and also support the use of statin drugs in diabetic patients. In addition, hepatic LRP1 has not been reported as degraded by proprotein convertase subtilisin kexin type 9, which is also regulated by SREBP-2 and degrades hepatic LDL receptors [36,37]. Therefore, we can expect additional antiatherogenic effects of high-dose statin treatment despite the limited effect of high-dose statin treatment on lowering plasma LDL due to co-upregulation of hepatic LDL receptor and proprotein convertase subtilisin kexin type 9 by statin drugs.

The limitation of this study is that the dose of atorvastatin was not a physiologic dose. We treated relatively high pharmacologic dose of atorvastatin for in vitro and in vivo studies. Further studies are needed to confirm our results as well as suggested mechanism using physiologic doses of HMG-CoA reductase inhibitors in humans. Nevertheless, the doses of the present study have been generally used in the studies investigating the effect of atorvastatin. In addition, hepatic SREBP-2 is believed to be increased by statin drugs in humans; and therefore, hepatic LRP1 expression could be increased by a standard dose of atorvastatin in humans according to our suggested mechanism including SREBP-2 regulation.

In conclusion, atorvastatin increased LRP1 expression in 2 hepatocyte-derived cell lines and in the liver of 2 animal models, This up-regulation of hepatic LRP1 might be a novel mechanism through which HMG-CoA reductase inhibitors clear circulating remnant lipoproteins. In addition, intracellular cholesterol level and SREBP-2 expression were associated with LRP1 expression in hepatocyte-derived cell lines; and SREBP-2 gene silencing using siRNA showed that SREBP-2 might act as an activator of the transcription of the hepatic *LRP1* gene.

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