Original Article

HMG-CoA Reductase Inhibitor, Simvastatin Improves Reverse Cholesterol Transport in Type 2 Diabetic Patients with Hyperlipidemia

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Aim: ApoA-I and HDL promote cellular cholesterol efflux in the early stages of the reverse cholesterol transport (RCT) pathway. A low plasma HDL-C level is characteristic of atherogenic dyslipidemia in patients with type 2 diabetes. We evaluated plasma lipid levels and the expression of factors related to RCT in type 2 diabetic patients, and the effects of an HMG-CoA reductase inhibitor, simvastatin, were studied.

Methods: Messenger RNA (mRNA) expression in circulating mononuclear cells was analyzed by reverse transcription-polymerase chain reaction (RT-PCR), focusing on the following factors: liver X receptor α (LXR α), ATP-binding cassette A1 (ABCA1), scavenger receptor class B type 1 (SR-B1), apolipoprotein E (ApoE), apolipoprotein A-1 (ApoA-1), caveolin, and cholesterol ester transfer protein (CETP). Type 2 diabetic subjects ($n=29$) were divided into three subgroups: patients with normolipidemia (DM group, $n=11$), patients with untreated hyperlipidemia (DMHL group, $n=10$), and those with hyperlipidemia treated with simvastatin 5-10 mg/day (DMST group, $n=8$). The control group (CNT group) included seven healthy volunteers.

Results: Simvastatin treatment significantly increased plasma levels of ApoA-I compared to the other three groups. Simvastatin treatment improved the expression of mRNA for LXR α , ABCA1, and ApoA-I compared with DMHL or control groups.

Conclusion: Our data suggest that RCT may be reduced in type 2 diabetic patients with hyperlipidemia, and simvastatin may be able to improve reverse cholesterol transport for this population of diabetic patients.

J Atheroscler Thromb, 2008; 15:20-25.

Key words; Statin, Reverse cholesterol transport, HDL-C, ABCA1

Introduction

The liver plays a major role in cholesterol metabolism (i.e. cholesterol synthesis), its degradation to bile acids, and elimination to bile. Most of the cholesterol that deposits in tissues must ultimately be transported to the liver by a process referred to as reverse cholesterol transport (RCT).

High-density lipoprotein (HDL) has been identified as taking a central role in the RCT pathway, although plasma levels of HDL-C are not necessary for RCT function. Low HDL-C levels, coexisting with elevated triglycerides and small-dense LDL particles, are characteristic of dyslipidemia in patients with type 2 diabetes¹⁾.

Recent reports suggested that HMG-CoA reductase inhibitors (i.e. statins) may have the potential to indirectly activate PPAR α ²⁾ and induce ApoA-I, leading to an elevation of plasma HDL^{2, 3)}; however, there have been few reports on the RCT pathway in diabetic patients and on the effects of statins on RCT in this

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Received: February 28, 2007

Accepted for publication: September 3, 2007

population^{4, 5)}.

In the present study, we examined mRNA levels of the key regulatory genes involved in RCT including liver X receptor α [LXR α], ATP-binding cassette A1 [ABCA1], scavenger receptor class B type 1 [SR-B1], apolipoprotein A-1 [ApoA-1], caveolin, and cholesterol ester transfer protein [CETP] from type 2 diabetes patients; the effects of statin were evaluated. Due to the difficulty of obtaining human liver samples, we measured mRNA levels in circulating monocytes under the hypothesis that these cells can be considered representative of macrophages⁴⁾ and hepatocytes⁶⁾ with respect to the expression of key regulatory genes of cholesterol metabolism.

Subjects and Methods

Patients

The study protocol and informed consent document were reviewed and approved by the Ethics Committee of Hirosaki University. After being fully informed regarding all aspects of their participation in this study, informed consent was obtained from all subjects. Inclusion criteria for the study required that the subjects' medications to treat diabetes and hyperlipidemia were unchanged for the 6 months prior to study entry. Diabetic patients who had been receiving insulin therapy were excluded from this study. For the 6 months prior to participation in this study, the condition of all diabetic subjects was stable in relation to the control of diabetes and the presence of hyperlipidemia; each subject's body mass index (BMI) remained virtually unchanged during this 6-month period.

The study subjects included 29 patients with type 2 diabetes and 7 healthy controls (CNT group). The control subjects were normal on physical examination, with plasma glucose and lipid levels within normal limits. The 29 diabetic patients were divided into the following three subgroups:

- DM group—11 patients with normal plasma lipid levels. Five of these patients were treated with diet alone, while three were treated with sulfonylurea and two were treated with an α -glucosidase inhibitor, with combination therapy in one patient.
- DMHL group—10 patients with hyperlipidemia for which they had not received any treatment. Three of these patients had their diabetes treated with diet alone, six patients were treated with sulfonylurea and a single patient was receiving combination therapy with sulfonylurea and an α -glucosidase inhibitor.
- DMST group—8 patients with hyperlipide-

mia were treated with simvastatin 5–10 mg/day. Five patients had their diabetes treated with diet alone, while three were treated with sulfonylurea.

Plasma Glucose and Lipid Levels

The diabetic subjects consumed a diet of 1,440–1,800 Kcal/day (25 Kcal/Kg/day). Blood was collected in order to measure fasting plasma glucose (FPG), HbA1c, and lipid concentrations. Fasting plasma glucose was measured using the glucose oxidase method with a glucose analyzer (GA-1160 analyzer; Arkray, Tokyo, Japan). Serum concentrations of total cholesterol (TC), HDL cholesterol, triglyceride (TG), and free fatty acids (FFA) were measured using enzymatic colorimetric methods⁷⁾ and an automated multianalyzer (TBA-200FR; Toshiba, Tokyo, Japan). HbA1c levels were quantified using high-performance liquid chromatography (HLC-723 GHbV; Toso, Tokyo, Japan), and low-density lipoprotein (LDL) cholesterol was calculated according to the equation of Friedewald.

Messenger RNA Levels in Blood Monocytes

Blood (2.5 mL) was collected in a blood RNA vacuum tube (PreAnalytiX; Giagen BD Company, UK); total RNA in circulating mononuclear cells was separated according to the manufacturer's specifications (PAXgeneTM Blood RNA Kit; PreAnalytiX). The levels of mRNA for LXR α , ABCA1, SR-B1, apolipoprotein E (ApoE), ApoA, caveolin, CETP, and β -actin were analyzed by reverse transcription-polymerase chain reaction (RT-PCR)⁴⁾. Synthesis of single-strand cDNA for a PCR template and amplification was performed using an RNA PCR Kit (Ver.2.1; TaKaRa, Kyoto, Japan). Specific primers were designed from cDNA sequences, as shown in **Table 1**. The protocol of the reaction conditions was 1 $\times$ (94°C, 2 min), 30 $\times$ (94°C, 30s; 55°C, 45s; 72°C, 120s), 1 $\times$ (72°C, 10 min), and 1 $\times$ (4°C, 5 min).

PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide; the bands were then visualized by UV-induced fluorescence. The expression of each mRNA was normalized by the expression of β -actin using Fragment Manager software (Pharmacia, Stockholm, Sweden).

Statistical Analysis

Data are expressed as the mean $\pm$ SE. Comparisons of values in control and diabetic groups were performed using one-way analysis of variance (ANOVA) with post-hoc Tukey's test. A *p*-value of <0.05 was regarded as significant.

Table 1. Sequences of the primers

mRNA	Sense primers	Antisense primers
Caveolin-1	ACAGACGGTGTGGACGTAGAT	GAGCGAGAAGCAAGTGTACGA
LXR α	ATGGACACCTACATGCGTC	ATGGTGGATGGAGACGTAG
ABCA1	CAGGAGGTGATGTTTCTGACCA	TTGGCTGTTCTCCATGAAGGTC
SRB1	TCGCTCATCAAGCAGGAGGT	GCCCAGAGTCGGAGTTGTTG
ApoE	CTTCATGGTCTCGTCCATCAGC	AAGGACGTCTTCCCCAGGAGC
ApoA-I	TGGGATCGAGTGAAGGACCT	CTCCTCCTGCCACTTCTTCTG
β -actin	TCCTTCTGCATCCTGTCGGCA	CAAGAGATGGCCACGGCTGCT
CETP	TGCTCCCCAAGAAGGGCTGACT	AGGAATCCTGTCTGGGCCTCTCT

Table 2. Clinical characteristics of subjects

Group	M/F (year)	Age (y.o.)	BMI (kg/m ²)	FPG (mg/dL)	HbA1c (%)	FFA (mEq/L)
CNT	4/3	65.4 $\pm$ 1.9	25.0 $\pm$ 0.8	91 $\pm$ 4.5*	5.1 $\pm$ 0.2*	612 $\pm$ 101
DM	5/6	68.4 $\pm$ 2.1	26.9 $\pm$ 0.6	140.4 $\pm$ 10.7	7.3 $\pm$ 0.6	767 $\pm$ 112
DMHL	5/5	69.8 $\pm$ 2.1	25.9 $\pm$ 1.1	134.5 $\pm$ 12.8	7.9 $\pm$ 0.7	799 $\pm$ 130
DMST	3/5	68.2 $\pm$ 1.9	29.1 $\pm$ 1.9	142.6 $\pm$ 10.9	6.6 $\pm$ 0.3	731 $\pm$ 99

Values are expressed as the mean $\pm$ SE.

* $p < 0.05$ compared to DM, DMHL and DMST groups.

CNT, control; DM, diabetic patients with normal plasma lipid levels; DMHL, diabetic patients with hyperlipidemia who had not received anti-hyperlipidemic treatment; DMST, diabetic patients with hyperlipidemia treated with simvastatin.

Table 3. Plasma lipid and apolipoprotein levels

Group	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	ApoA-I (mg/dL)	ApoB (mg/dL)	ApoE (mg/dL)
CNT	174 $\pm$ 4	97 $\pm$ 12	58.0 $\pm$ 7.8	97 $\pm$ 8	135 $\pm$ 16	79 $\pm$ 7	3.9 $\pm$ 0.3
DM	178 $\pm$ 8	115 $\pm$ 18	50.1 $\pm$ 1.8	109 $\pm$ 9	130 $\pm$ 5	94 $\pm$ 5	3.9 $\pm$ 0.3
DMHL	239 $\pm$ 4 ^{a, b}	152 $\pm$ 28	50.5 $\pm$ 3.7	148 $\pm$ 8 ^{a, b}	136 $\pm$ 9	121 $\pm$ 3 ^{a, b}	4.9 $\pm$ 0.4 ^{a, b}
DMST	222 $\pm$ 9 ^{a, b}	136 $\pm$ 24	57.3 $\pm$ 3.7	123 $\pm$ 16	157 $\pm$ 9 ^b	113 $\pm$ 12 ^a	4.8 $\pm$ 0.5

Values are expressed as the mean $\pm$ SE.

^a $p < 0.05$ compared to the CNT group. ^b $p < 0.05$ compared to the DM group. $p < 0.05$, significantly different from the DM value.

CNT, control; DM, diabetic patients with normal plasma lipid levels; DMHL, diabetic patients with hyperlipidemia who had not received anti-hyperlipidemic treatment; DMST, diabetic patients with hyperlipidemia treated with simvastatin.

Results

Baseline Characteristics

The baseline clinical features of all subjects are shown in **Table 2**. As anticipated, fasting plasma glucose and HbA1c levels were significantly elevated in diabetic subjects as compared to controls.

Plasma Lipid and Apolipoprotein Levels

Table 3 shows the plasma lipid and apolipoprotein levels in each of the subject groups. Plasma levels of triglycerides, LDL-C, ApoB, and ApoE in the DMHL group were significantly elevated compared to the DM and control groups. HDL-C levels were not significantly different between groups, although the

mean values in the DM (50.1 mg/dL) and DMHL (50.5 mg/dL) groups were lower than the controls (58.0 mg/dL). Furthermore, there was tendency toward higher HDL-C levels in subjects in the DMST (57.3 mg/dL) group. ApoA-I levels in the DMST group were significantly higher than in the DM group (157 mg/dL vs. 130 mg/dL, respectively).

Messenger RNA Expression of Key Regulatory Genes Involved in Reverse Cholesterol Transport in Circulating Monocytes

DMHL subjects showed a trend towards a decrease in LXR α and ABCA1 mRNA expression compared to DM subjects (**Fig. 1A, B**).

LXR α , ABCA1 and ApoA-I mRNA levels in

Fig. 1. Expression of mRNA for key regulatory genes involved in reverse cholesterol transport.

Expression of mRNA for LXRα (A), ABCA1 (B), ApoA-I (C), and ApoE (D) in peripheral blood mononuclear cells was analyzed by RT-PCR. Expression of each mRNA was normalized by β-actin. CNT, control; DM, diabetic patients with normal plasma lipid levels; DMHL, diabetic patients with hyperlipidemia who had not received anti-hyperlipidemic treatment; DMST, diabetic patients with hyperlipidemia treated with simvastatin. * $p < 0.05$.

DMST subjects were significantly elevated compared to DMHL or DM subjects (**Fig. 1A, B, C**). LXRα mRNA levels were improved by simvastatin treatment (DMST; 0.74) compared to levels found in DMHL (0.52) and control (0.56) groups (**Fig. 1A**). Similarly, simvastatin treatment increased mRNA levels of ABCA1 (0.35) compared to DM (0.26) and DMHL (0.18) groups (**Fig. 1B**). ApoA-I mRNA levels were also increased in DMST (0.79) compared to levels in control (0.59), DM (0.68), and DMHL (0.70) groups (**Fig. 1C**). ApoE mRNA levels were significantly reduced in the DMHL group (0.72) compared to control subjects (0.88) (**Fig. 1D**); however, those were not changed by statin treatment.

Caveolin, CETP, and SR-B1 mRNA levels were comparable between control and all diabetic groups (data not shown).

Discussion

Both ApoA-I and HDL promote cellular chole-

sterol efflux via ATP-binding cassette transporters (ABCA1 and ABCG1); this is the early stage of RCT, when cholesterol is removed from extrahepatic tissues and transferred to the liver⁸⁻¹¹. Scavenger receptor class B type 1 may also be involved in the cellular efflux of cholesterol and play a key role in RCT¹². The ABC group of transporters and SR-B1 are concentrated in the caveolae of the hepatocyte cell surface^{13, 14}. Caveolae function in the transduction of cell surface signals and in the regulation of cholesterol homeostasis; caveolin is an important protein constituent of the caveolae^{15, 16}.

Lecithin: cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein are also important factors (enzymes) in the RCT system. Esterification of free cholesterol by LCAT contributes to HDL maturation. Subsequently, CETP action enables the transfer of cholesteryl ester from HDL to lipoproteins of lower density, making HDL particles cholesteryl ester-depleted and triglyceride-enriched^{17, 18}.

Epidemiological studies have indicated an inverse

relationship between the HDL-C level and cardiovascular disease¹⁹⁻²². Plasma HDL-C levels are not necessarily indicators of effective reverse cholesterol transport, but the kinetics of HDL metabolism and transport of lipids are thought to be important in this process^{11, 22}.

It is worth studying RCT function in type 2 diabetic patients, because low plasma HDL-C levels associated with high plasma triglyceride levels are well-known features of dyslipidemia in type 2 diabetes mellitus. We examined mRNA levels of the key regulatory genes in RCT (i.e., LXR α , ABCA1, ApoA-I, CETP, SR-B1, and caveolin) from patients with type 2 diabetes. It is still controversial whether this early step in the RCT process is affected in type 2 diabetes. Some studies suggest that *in vitro* glycation of HDL impairs its ability to promote cholesterol efflux from human skin fibroblasts²³. On the other hand, Vries *et al.* concluded that there was no primary defect in the early process in the RCT pathway in type 2 diabetes using cultured fibroblasts²⁴. Recently, Mauldin *et al.* reported selective loss of ABCG1-mediated efflux to HDL and demonstrated foam cell formation in mice with type 2 diabetes²⁵.

Hepatocytes and macrophages are adequate samples for the evaluation of RCT; however, collecting these specimens for evaluation is not practical in human subjects. Thus, we analyzed the expression of genes in circulating mononuclear leukocytes that would become macrophages in peripheral tissues (including vascular vessel walls). Powell *et al.* reported that the basic regulatory mechanisms in liver and mononuclear leukocytes are similar⁶.

Statins are inhibitors of the rate-limiting enzyme in cholesterol synthesis (i.e., 3-hydroxy-3-methylglutaryl coenzyme A reductase), and serve to reduce endogenous cholesterol synthesis. Low levels of endogenous cholesterol activate sterol regulatory element-binding protein, which increases the expression of LDL receptors and leads to the lowering of plasma LDL²⁶. Although the focus of anti-hyperlipidemic therapy with statins has been the lowering of LDL-C levels, several statins (including simvastatin) are approved for the treatment of low HDL levels²⁷, but there have been few well-controlled studies on statin effects on HDL-C levels, including evaluation of the effects in patients with diabetes^{4, 5, 28}. Martin *et al.* demonstrated that statins activate PPAR α through inhibition of the Rho A signal transduction pathway, and activation of the PPAR α -LXR α -ABCA1 pathway may enhance plasma HDL levels, particularly constituent ApoA-I².

In the present study, we demonstrated that diabetic patients with hyperlipidemia had significantly re-

duced expression of ApoE mRNA in circulating mononuclear cells compared to diabetic patients with normolipidemia. We found no evidence for the increased expression of SR-B1 and caveolin in circulating mononuclear cells, consistent with previous findings⁵.

Therapeutic approaches to enhance RCT would be novel treatment for atherosclerotic diseases, including diabetes. Simvastatin treatment significantly increased plasma ApoA-I levels compared to the levels seen in study subjects that had not been administered this drug. Simvastatin-treated subjects showed enhanced mRNA expression for LXR α , ABCA1, and ApoA-I compared with subjects in DMHL or DM groups. Simvastatin has been reported to decrease circulating levels of CETP^{29, 30}; however, in our study, the expression of CETP mRNA was unchanged in the DMST group's circulating mononuclear cells.

Our data suggest that RCT may be reduced in type 2 diabetic patients with dyslipidemia, and simvastatin may be able to improve the factors related to reverse cholesterol transport. Many factors and enzymes are involved in RCT, and further studies are necessary to evaluate every stage of the pathway.

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