

ORIGINAL

Ezetimibe, an inhibitor of Niemann-Pick C1-like 1 protein, decreases cholesteryl ester transfer protein in type 2 diabetes mellitus

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Abstract. To address the effects of ezetimibe on high-density lipoprotein (HDL) metabolism, the HDL subclasses, cholesteryl ester transfer protein (CETP), and lecithin-cholesterol acyltransferase (LCAT) were measured in patients with type 2 diabetes mellitus (T2DM). Twenty-three hypercholesterolemic patients with T2DM were treated with 10 mg of ezetimibe daily for 12 weeks. Plasma total cholesterol (TC), low-density lipoprotein (LDL)-cholesterol (C), HDL-C, HDL₂-C, HDL₃-C, CETP mass, and LCAT activity were measured. HDL-C and HDL₂-C increased by 5% ($p<0.05$) and 12% ($p<0.01$), respectively, in response to ezetimibe. Of the 23 patients, 21 had decreased CETP mass, which led to an average reduction of 20% ($p<0.0001$). LCAT activity also decreased by 6% ($p<0.01$). A significant positive correlation was found in the changes from baseline between HDL₂-C and CETP mass, whereas a significant inverse relationship was observed between HDL₃-C and CETP mass. Furthermore, the change in HDL-C was positively correlated with the change in LCAT activity. In conclusion, ezetimibe may affect HDL metabolism and reverse cholesterol transport, especially CETP, in T2DM. These observations may provide some insights into how ezetimibe prevents atherosclerosis.

Key words: Ezetimibe, Type 2 diabetes, High-density lipoproteins, Lecithin-cholesterol acyltransferase, Cholesteryl ester transfer protein

EZETIMIBE is a drug that lowers plasma low-density lipoprotein (LDL)-cholesterol (C) and non-high-density lipoprotein (HDL)-C by inhibiting intestinal cholesterol absorption [1, 2]. It has been proposed that ezetimibe inhibits Niemann-Pick C1-like 1 (NPC1L1) protein [3], which is highly expressed in the brush border membrane of the enterocyte, where it facilitates intestinal cholesterol absorption. Furthermore, humans, but not mice, also express NPC1L1 in the liver as much as in the small intestine [3, 4], suggesting the essential role of hepatic NPC1L1 in hepatic and plasma lipid metabolism. In fact, ezetimibe ameliorates non-alcoholic steatohepatitis in humans [5].

Elevated LDL-C and decreased HDL-C levels are

conventional risk factors for cardiovascular disease in patients with type 2 diabetes mellitus (T2DM). Ezetimibe has been shown to be effective in reducing plasma LDL-C and non-HDL-C levels in hypercholesterolemic patients with T2DM [6-8]. In addition, a meta-analysis of randomized, controlled trials in which diabetic patients accounted for 2-8% of the patients also showed that ezetimibe monotherapy significantly increased plasma HDL-C levels by 3.0% in patients with primary hypercholesterolemia [9], though the precise mechanism has not yet been determined.

Lecithin-cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP) are key enzymes in the reverse cholesterol transport system and are involved in HDL metabolism [10, 11]. LCAT converts free cholesterol into cholesteryl esters (CEs), which are then sequestered into the core of lipoprotein particles, making the spherical HDL₃ particles that are converted into HDL₂ particles by the phospholipid transfer protein (PLTP)-activated fusion of smaller

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HDL particles. CETP mediates neutral transport of CEs and triglycerides (TGs) between lipoproteins, resulting in the transfer of CEs in exchange for TGs from HDL to atherogenic lipoproteins such as LDL and very low-density lipoprotein (VLDL). TGs accumulating in HDL are then hydrolyzed by hepatic lipase (HL). Moreover, CETP transfers CEs between HDL particles and generates HDL₂ and pre- β HDL from HDL₃ particles.

We recently reported that pitavastatin decreases CETP mass and LCAT activity in hypercholesterolemic patients [12]. Furthermore, genome-wide association studies involving >100,000 individuals of European ancestry identified LCAT and CETP as significantly associated loci with plasma HDL-C [13]. In the present study, the effects of ezetimibe on the HDL subclasses, CETP mass, and LCAT activity were assessed in T2DM, and their relationships were examined.

Subjects and Methods

Hypercholesterolemic patients with T2DM ($n = 23$; men/women = 13/10, age = 59.1 ± 9.5 years) were enrolled in the study (Table 1). They received sulfonylureas ($n=11$), glinides ($n=3$), pioglitazone ($n=3$), biguanides ($n=6$), α -glucosidase inhibitors ($n=2$), and dipeptidyl peptidase-4 inhibitors ($n=1$). According to the plasma LDL-C levels as recommended by the guidelines of the Japan Atherosclerosis Society [14], patients with plasma LDL-C >120 mg/dL were the sub-

jects of this study. None of the patients were taking any lipid-lowering drugs such as statins and fibrates. The patients were given 10 mg of ezetimibe daily for 12 weeks, while other drugs for other diseases, such as diabetes and hypertension, were left unchanged. The study was approved by the Ethics Committee of Jichi Medical University, and all patients gave their written, informed consent.

At baseline and after 12 weeks of treatment, blood samples were collected after a 12-h fasting period, and the following parameters were determined in plasma: total cholesterol (TC), TGs, HDL-C, HDL₂-C, HDL₃-C, LCAT activity, CETP mass, sitosterol, campesterol, cholestanol, lathosterol, high-sensitivity C-reactive protein (hs-CRP), glucose, and hemoglobin A1c (HbA1c).

Plasma TC, TGs, and HDL-C were measured by automated enzymatic assays. LDL-C levels were calculated by the Friedewald formula, since all patients had TG levels <400 mg/dL. HDL₂ and HDL₃ were isolated by density gradient ultracentrifugation, and the cholesterol levels in these lipoproteins were measured enzymatically. CETP mass was measured by ELISA assay (CETP ELIZA-DAIICHI, Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). LCAT activity was measured using dipalmitoyl lecithin as the substrate [15]. Sitosterol, campesterol, cholestanol, and lathosterol were measured by gas-liquid chromatography [16]. The hs-CRP level was determined using an

Table 1 Patients' characteristic at baseline and after 12 weeks of ezetimibe treatment

parameter	at baseline	after 12 weeks
Sex (Men/women)	10/13	-
Age	59.1 ± 9.5	-
TC (mg/dL)	232.7 ± 33.9	$201.6 \pm 28.1^{***}$
TG (mg/dL)	127.8 ± 54.3	$118.65 \pm 67.3^{***}$
LDL-C (mg/dL)	152.8 ± 26.8	$121.0 \pm 20.0^{***}$
HDL-C (mg/dL)	53.7 ± 13.4	$56.4 \pm 17.1^*$
HDL ₂ -C (mg/dL)	33.1 ± 11.0	$37.1 \pm 14.4^{**}$
HDL ₃ -C (mg/dL)	19.8 ± 4.1	20.0 ± 3.3
LCAT (U)	116.3 ± 15.5	$108.8 \pm 14.0^{**}$
CETP (μ g/mL)	2.5 ± 0.5	$2.0 \pm 0.5^{***}$
Sitosterol (μ g/mL)	3.9 ± 1.5	$2.0 \pm 0.8^{***}$
Campesterol (μ g/mL)	6.8 ± 2.8	$3.2 \pm 1.3^{***}$
Cholestanol (μ g/mL)	3.0 ± 0.7	$2.7 \pm 0.5^*$
Lathosterol (μ g/mL)	4.4 ± 2.4	4.9 ± 2.1
Glucose (mg/mL)	128.0 ± 27.4	136.6 ± 35.4
HbA1c (%)	7.1 ± 0.8	7.2 ± 0.9
Hs-CRP (ng/mL)	100.0 ± 277.8	58.4 ± 99.2

Results were expressed as mean \pm S.D. *** $p < 0.0001$, * $p < 0.05$, ** $p < 0.01$ vs. baseline

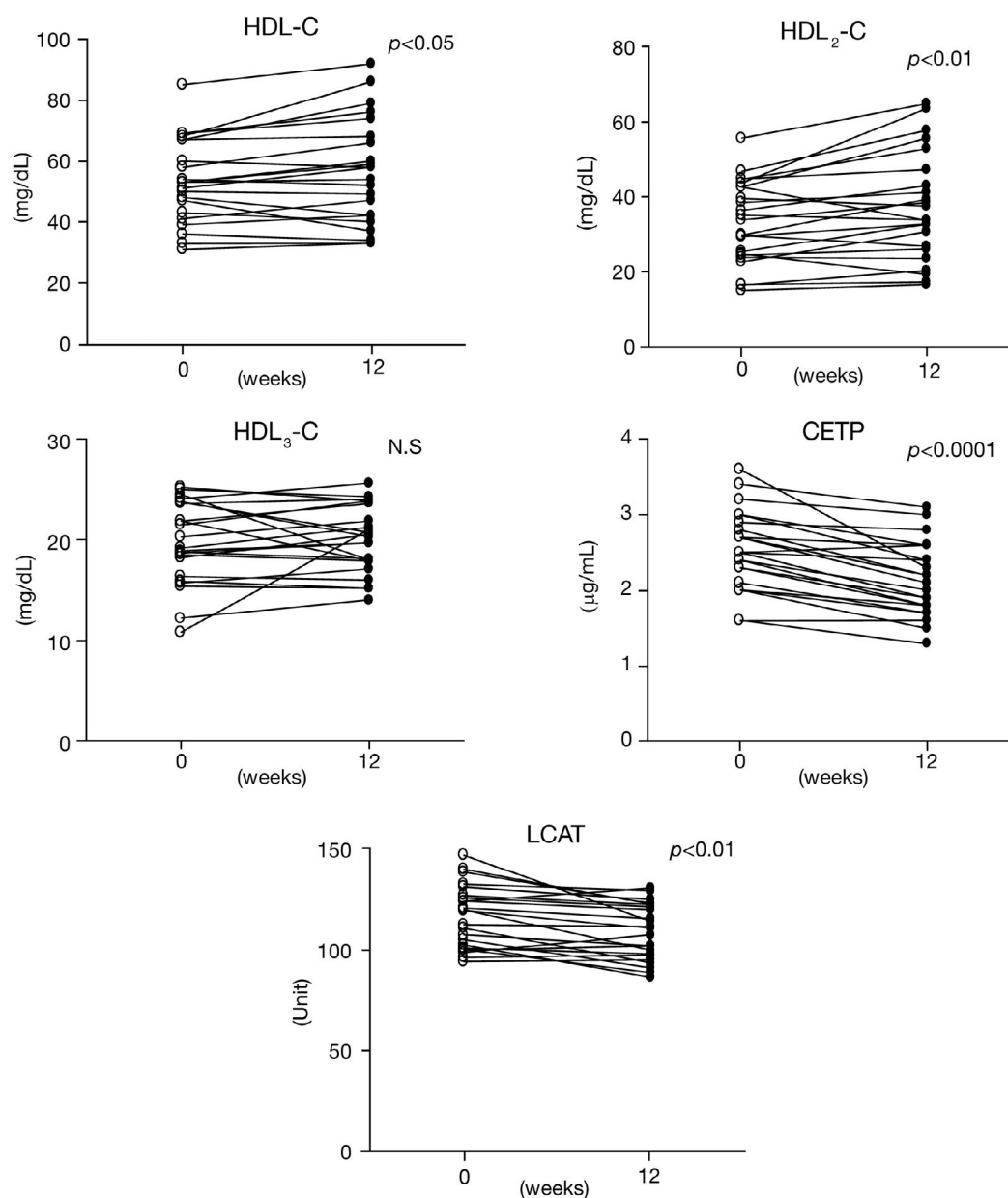


Fig. 1 HDL subclasses, CETP mass, and LCAT activity at baseline and at 12 weeks of ezetimibe treatment. Ezetimibe (10 mg/daily) was given for 12 weeks, and blood was collected before and after ezetimibe treatment. Plasma HDL-C, HDL₂-C, HDL₃-C, CETP mass, and LCAT activity were measured and compared before and after treatment. Data are expressed as means \pm S.D. N.S: not significant.

ultra-high-sensitivity latex turbidimetric immunoassay (Bering Nephelometry, Tokyo, Japan).

Student's paired *t*-test was used for statistical analyses. Univariate Pearson's correlation coefficient analysis was performed to estimate the relationship between two variables. A *p* value of <0.05 was considered significant.

Results

Plasma TC and LDL-C levels decreased by 13% ($p < 0.0001$) and 21% ($p < 0.0001$), respectively, when patients were treated with ezetimibe for 12 weeks (Table 1, Fig. 1). Ezetimibe did not affect TG levels. HDL-C and HDL₂-C levels were marginally but significantly

increased after treatment. The cholesterol absorption markers, sitosterol, campesterol, and cholestanol, were significantly decreased by 49%, 53%, and 10%, respectively (Table 1). In contrast, no significant change was observed in lathosterol, a marker of cholesterol synthesis. There was no significant relationship in the changes from baseline between each cholesterol synthesis or absorption marker and LDL-C levels. The changes in HDL-C and HDL₂-C also did not have a significant relationship with the changes in any cholesterol absorption markers. The change in HDL₃-C had a significant positive correlation with the change in campesterol ($p < 0.01$), but not sitosterol and cholestanol (data not shown).

After 12 weeks of treatment, 21 of the 23 patients had decreased CETP mass, which led to an average reduction of 20% ($p < 0.0001$) (Table 1, Fig. 1). A sig-

nificant positive correlation was found in the changes from baseline between HDL₂-C levels and CETP mass, whereas a significant inverse relationship was observed in the changes from baseline between HDL₃-C levels and CETP mass (Fig. 2). The change in HDL-C levels was not associated with that in CETP mass. LCAT activity was also decreased by 6% ($p < 0.01$) with ezetimibe (Table 1, Fig. 1). A significant positive correlation was observed in the change from baseline between LCAT activity and HDL-C, but not HDL₂-C or HDL₃-C levels (Fig. 3). The change in LDL-C was not associated with the change in CETP mass or LCAT activity (data not shown).

Plasma hs-CRP, glucose, and HbA1c levels were not affected by ezetimibe therapy.

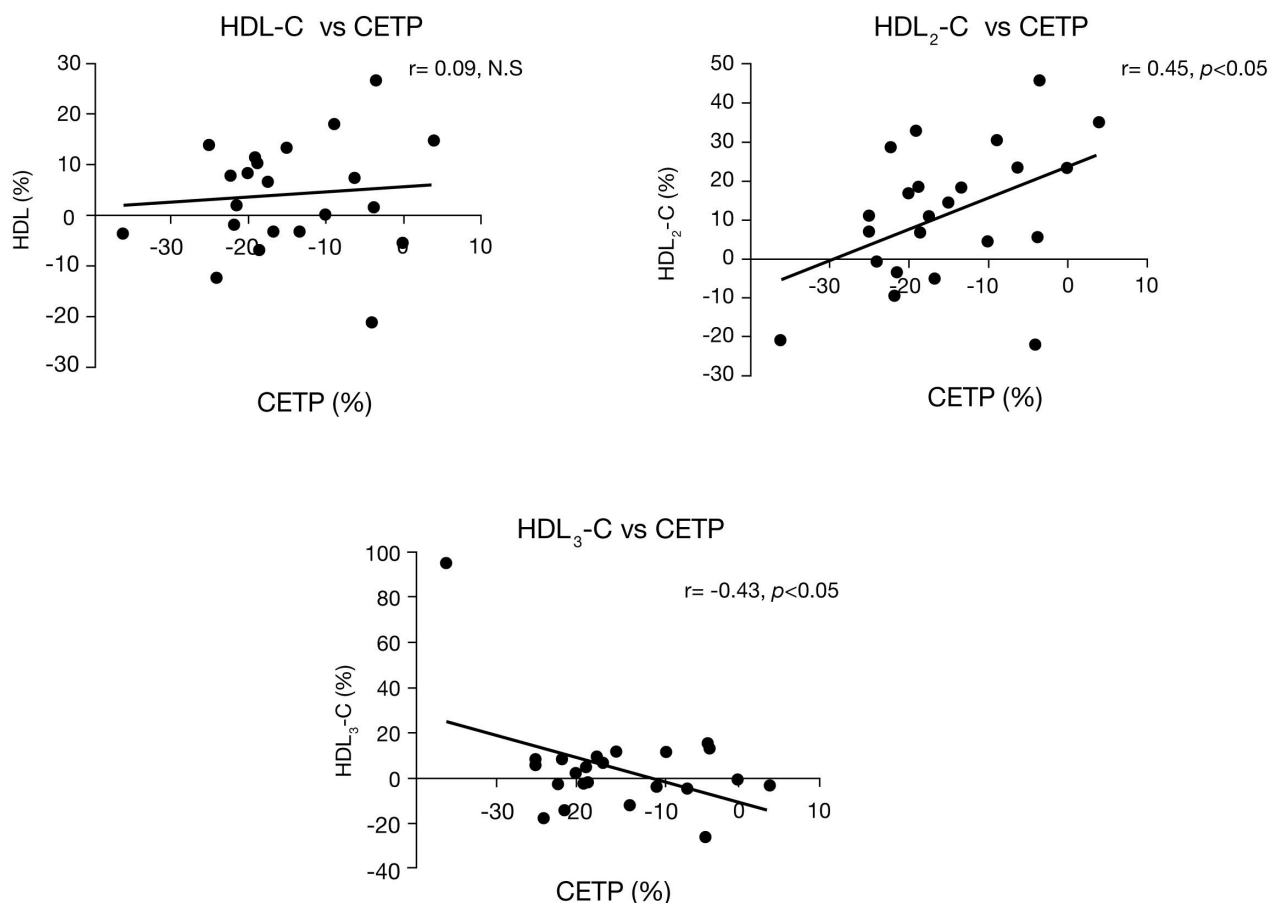


Fig. 2 Relationships between HDL subclasses and CETP mass. Ezetimibe (10 mg/daily) was given for 12 weeks, and blood was collected before and after ezetimibe treatment. The percent changes in HDL subclasses and CETP mass in response to ezetimibe treatment were calculated, and their relationships were examined. Data are expressed as means \pm S.D. N.S: not significant.

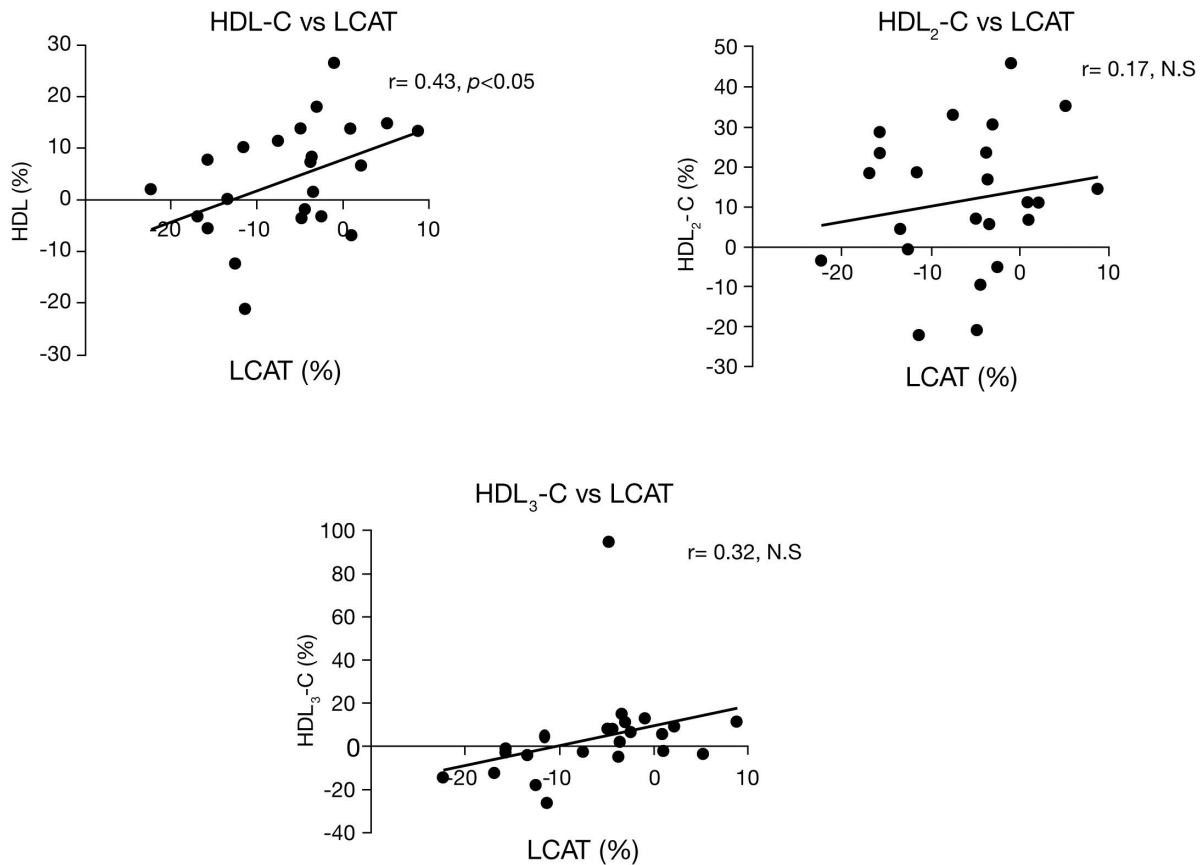


Fig. 3 Relationships between HDL subclasses and LCAT activity. Ezetimibe (10 mg/daily) was given for 12 weeks, and blood was collected before and after ezetimibe treatment. The percent changes in HDL subclasses and LCAT activity in response to ezetimibe treatment were calculated, and their relationships were compared. Data are expressed as means \pm S.D. N.S: not significant.

Discussion

This is the first study to demonstrate the effects of ezetimibe on HDL subclasses, CETP mass, and LCAT activity in hypercholesterolemic patients with T2DM. Because these patients had not used any other drugs for hyperlipidemia, the observations in the present study simply reflect the effects of ezetimibe.

As expected, ezetimibe significantly reduced plasma TC and LDL-C levels. Markers for cholesterol absorption were also significantly decreased, but lathosterol, a marker of cholesterol synthesis, was not affected by ezetimibe therapy. In contrast to a study in which there was a positive correlation in the changes between a cholesterol absorption/synthesis marker and LDL-C in response to ezetimibe treatment [7], such a correlation was not observed in the present study. The reason for this discrepancy is unclear, but the results of the present study imply that the LDL-lowering effect of ezetimibe

is not simply determined by the prevention of cholesterol absorption in T2DM. The reduction of CETP and LCAT by ezetimibe may also affect LDL metabolism, though a significant correlation was not found in the changes from baseline between CETP mass or LCAT activity and LDL-C.

In the present study, it was demonstrated that HDL-C and HDL₂-C, but not HDL₃-C, were significantly increased at 12 weeks of ezetimibe administration. Because HDL-C is 10–20% lower in T2DM [17], which is mainly the result of a decrease in HDL₂ and to some extent in HDL₃, ezetimibe is the drug to improve the decreased HDL levels in T2DM. Why ezetimibe modulates HDL metabolism remains unclear. It has been reported that plasma HDL-C levels are positively correlated with markers of cholesterol absorption in metabolic syndrome cases and healthy individuals [18, 19]. In the present study, the change in HDL₃-C was significantly associated with the change in campesterol.

However, the change in HDL₃-C was not associated with the changes in other cholesterol absorption markers such as sitosterol and cholestanol. Furthermore, the changes in HDL-C and HDL₂-C were not associated with changes in any cholesterol synthesis markers. Taken together with the observation that the changes in LDL-C was not associated with the changes in any cholesterol absorption markers, ezetimibe may be involved in plasma lipoprotein metabolism by means of not only intestinal but also hepatic lipid metabolism, because humans express NPC1L1, a target of ezetimibe, in the liver as much as in the small intestine.

Interestingly, the change in HDL₂-C levels was positively correlated with that in plasma CETP mass, whereas an inverse relationship was observed in the change between HDL₃-C levels and CETP mass (Fig. 2). Moreover, the change in HDL-C was positively correlated with the change in LCAT activity (Fig. 3). With regard to the relationship between CETP and HDL₂-C or HDL₃-C in the present study, these observations are contrary to the function of CETP, in which inhibition of CETP causes an imbalance between PLTP and CETP and results in the generation of larger HDL particles such as HDL₂, whereas smaller HDL particles such as HDL₃ are diminished [10]. Thus, these observations must be interpreted carefully. Indeed, the significant relationships in the changes between CETP and HDL₂-C or HDL₃-C disappeared after exclusion of one patient who had extremely high HDL₃-C elevations with ezetimibe. Moreover, in addition to HDL-C, the change in HDL₃-C came to have a significant positive correlation with the change in LCAT activity ($p < 0.01$) after exclusion of the patient who had extremely high HDL₃-C elevations with ezetimibe. Because many other proteins, e.g., HL and PLTP, are also involved in reverse cholesterol transport and HDL metabolism [11, 17], ezetimibe may affect these proteins, which acted in concert in HDL₂ and HDL₃ remodeling in the present study.

It should be noted that 91% of the patients had decreased CETP mass by an average of 20% in response to ezetimibe. This may simply reflect the effects of lipid-lowering drugs, because statins also decrease CETP mass in hypercholesterolemic patients [12]. Alternatively, ezetimibe may regulate the CETP gene at transcriptional levels. The CETP gene has sterol regula-

tory elements (SREs) in the promoter where sterol regulatory element-binding proteins (SREBPs) bind and then activate the CETP gene [20, 21]. The CETP gene has been shown to be activated by SREBP-1a rather than SREBP-2 in the liver and human liposarcoma cells, an adipocytic cell line [20, 21]. Furthermore, ezetimibe upregulates hepatic SREBP-2 and down-regulates hepatic SREBP-1c in wild-type mice fed a high-fat diet for 10 weeks [22]. Taken together, this suggests that ezetimibe suppresses CETP gene expression by modulating hepatic SREBPs. The mechanism by which ezetimibe decreased plasma LCAT activity in the present study is still unknown. This is simply secondary to the reduction of CETP by ezetimibe. Alternatively, ezetimibe may affect the gene expression levels of hepatic LCAT. Indeed, fibrates, which are drugs for the treatment of hyperlipidemia, have been shown to decrease hepatic LCAT mRNA at the transcriptional level, thereby reducing plasma LCAT activity [23]. Further study will be needed to determine whether ezetimibe decreases hepatic LCAT mRNA.

The role of ezetimibe in the prevention of atherosclerosis is still a matter of debate [24-26]. In the SEAS and ENHANCE trials, ezetimibe did not reduce the composite outcome of combined aortic valve events and ischemic events or carotid-artery intima-media thickness in patients with familial hypercholesterolemia [25, 26]. In contrast, reduction of plasma LDL-C levels by the combination of simvastatin and ezetimibe decreased the incidence of major atherosclerotic events in patients with advanced chronic disease in the SHARP trial [24]. Taken together with the fact that a CETP inhibitor has attracted attention as an anti-atherosclerotic agent [27, 28], ezetimibe may suppress atherosclerosis by means of its inhibitory effect on CETP.

In conclusion, ezetimibe modulates HDL metabolism and reverse cholesterol transport, especially CETP, in T2DM. These effects of ezetimibe, beyond the well-known effects on LDL, may provide some insights into how ezetimibe prevents atherosclerosis in humans.

Conflict of Interest

The authors have no further conflicts of interest to disclose.

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