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ORIGINAL ARTICLE

Ezetimibe improves postprandial hyperlipidaemia in patients with type IIb hyperlipidaemia

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ABSTRACT

Background Postprandial hyperlipidaemia is known to be a high-risk factor for atherosclerotic disease because of rapid and lasting accumulations of triglyceride-rich lipoproteins and remnants. The Niemann-Pick C1-Like 1 (NPC1L1) protein acts as an intestinal cholesterol transporter and ezetimibe, which inhibits NPC1L1, has been used in patients with hypercholesterolaemia. We investigated effects of ezetimibe on fasting lipid and lipoprotein profiles and postprandial hyperlipidaemia in patients with type IIb hyperlipidaemia.

Materials and methods Ezetimibe 10 mg per day was administered in ten patients with type IIb hyperlipidaemia for 2 months, and lipid and lipoprotein profiles were examined during fasting and after an oral fat loading (OFL) test.

Results In the fasting state, ezetimibe significantly decreased not only total cholesterol, low density lipoprotein (LDL)-cholesterol and apolipoproteinB-100 (apoB-100) levels but triglycerides (TG), apoB-48 and remnant lipoprotein cholesterol (RemL-C) levels. High performance liquid chromatography analysis showed that ezetimibe decreased cholesterol and TG levels in the very low density lipoprotein (VLDL) and LDL size ranges as well as apoB-100 levels, suggesting a decrease in numbers of VLDL and LDL particles. After OFL, ezetimibe decreased the area under the curve for TG, apoB-48 and RemL-C. Ezetimibe decreased postprandial elevations of cholesterol and TG levels in the chylomicrons (CM) size range, suggesting that the postprandial production of CM particles was suppressed by ezetimibe.

Conclusions These findings suggest that ezetimibe improves fasting lipoprotein profiles and postprandial hyperlipidaemia by suppressing intestinal CM production in patients with type IIb hyperlipidaemia and such treatment may prove to be effective in reducing atherosclerosis.

Keywords Apolipoprotein B-48, atherosclerosis, ezetimibe, postprandial hyperlipidaemia, remnants, triglycerides-rich lipoproteins.

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Introduction

Plasma triglycerides (TG) are mainly found in triglyceride-rich lipoproteins (TRL) consisting of chylomicrons (CM) and very low density lipoproteins (VLDL). TRL constitute a population of particles of heterogeneous size, origin and apolipoprotein (apo) and lipid content. CM assemble dietary cholesterol, TG and apoB-48 in enterocytes and VLDL assemble endogenous hepatic TG, cholesterol and apoB-100 in hepatocytes. These lipoprotein particles undergo partial hydrolysis predominantly by lipoprotein lipase (LPL) into smaller and more dense particles know as remnants, which are believed to be more atherogenic than the larger TRL. CM are produced in enterocytes, primarily through the use of exogenous lipid sources and apoB-48 recruitment and are secreted into thoracic lymph, from which they flow into the systemic circulation. LPL hydrolyses CM-TG to free fatty acids (FFA), and residual particles become CM remnants (CM-R) which are taken up by the liver via remnant receptors. VLDL assemble endogenous hepatic TG, cholesterol and apoB-100 in hepatocytes, which are secreted directly into the blood stream. There, LPL hydrolyses VLDL-TG to FFA, and residual particles become VLDL remnants. The liver takes up VLDL remnants and further hydrolysed particles, and the low density lipoproteins (LDL) are taken up via LDL receptors while these particles are supplying energy and lipids to peripheral tissues. In the postprandial state, blood levels of

CM and CM-R quickly rise to reflect the increased exogenous lipid supply. This subsequently activates endogenous lipid synthesis in the liver by increasing the hepatic lipid inflow, leading to augmented hepatic VLDL production. Postprandial hypertriglyceridaemia is caused by overproduction and/or impaired clearance of TRL and TRL remnants, leading to rapid accumulation and sustained blood levels after dietary intake. Both fasting and postprandial hypertriglyceridaemia are known to be risk factors for coronary heart disease [1,2].

Recently Niemann-Pick C1 Like 1 (NPC1L1) protein has been reported to play a central role in cholesterol absorption in enterocytes [3,4]. Genetic inactivation of NPC1L1 protein decreases cholesterol levels and atherosclerotic lesions in hyperlipidaemic apoE knockout mice fed a western diet [5,6]. Ezetimibe, a novel lipid-lowering compound, selectively inhibits intestinal cholesterol absorption by binding to NPC1L1 protein, reducing total cholesterol (TC) and TG levels and also reducing the development of atherosclerosis in apoE knockout mice [7,8]. Clinically, it has already been shown that administration of ezetimibe diminished fasting levels of total and LDL-cholesterol in patients with primary hypercholesterolemia in Japan and the United States [9,10]. Due to the nature of its medicinal properties, the investigation into the pharmacological effects of ezetimibe has focused primarily on the metabolism of sterols, including cholesterol, rather than on TG or TRL. However, ezetimibe has been reported to decrease fasting TG levels significantly in patients with combined hyperlipidaemia [10], and its underlying mechanism of action has not yet been elucidated. As fasting and postprandial TG levels are closely related, it is essential to understand the effects of ezetimibe in combined hyperlipidaemic patients with reference to postprandial TRL and remnant metabolism. In this study, we administered ezetimibe 10 mg day^{-1} orally to 10 patients with type IIb hyperlipidaemia who have both hypercholesterolaemia and hypertriglyceridaemia, and used oral fat loading (OFL) tests to evaluate changes in fasting and postprandial lipid and lipoprotein profiles.

Materials and methods

Subjects

Ten Japanese patients (two female, eight male) were enrolled in this study. All patients had been diagnosed with type IIb hyperlipidaemia according to the Japanese criteria (fasting TC level ≥220 mg dL^{-1} and fasting TG level ≥150 mg dL^{-1}). Ezetimibe (Bayer Yakuhin Ltd. (Tokyo, Japan) and Schering-Plough K.K. (Tokyo, Japan)) 10 mg was administered once daily to all patients for 2 months. None of the patients took any other drugs that might affect lipid or lipoprotein metabolism. Every medication other than ezetimibe was continued unchanged throughout the study period. Total calorie intake and composition of the diet were kept constant for each patient. All subjects gave written informed consent before participating in this study, and the ethics committee of the Osaka University Hospital approved the study design.

Measurement of serum samples

Fasting blood samples were drawn from each of the 10 enrolled patients before the start and after the conclusion of ezetimibe administration. Serum was separated by low-speed centrifugation (1200 g, 15 min, at 4°C) and stored at 4°C until measurement within a week. All samples were treated in accordance with the Helsinki Declaration. Concentrations of TC, TG and FFA were measured using the enzymatic method. Concentrations of LDL-cholesterol (LDL-C) and high density lipoprotein cholesterol were measured using the direct method. Concentrations of apoAI, AII, B, CII, CIII, and E were measured using the immunoturbidity method. Concentrations of high sensitivity C-reactive protein were measured using the immunonephelometric assay (Sekisui Medical Co., Ltd., Tokyo, Japan). Haemoglobin A1c levels were measured using high performance liquid chromatography (HPLC) method. Fasting plasma glucose levels were measured using a hexokinase UV method. Concentrations of fasting plasma insulin were measured using a chemiluminescent enzyme immunoassay (CLEIA) method (SRL Inc., Tokyo, Japan). HOMA-IR (homeostasis model assessment of insulin resistance) index was calculated as [fasting plasma insulin (μIU mL^{-1}) × fasting plasma glucose (mg dL^{-1})]/405. Concentrations of apoB-48 were measured using a sandwich CLEIA (Fuji Rebio Inc., Tokyo, Japan) [11]. Remnant lipoprotein cholesterol (RemL-C) levels were measured using a RemL-C homogenous assay, RemL-C (Kyowa Medex, Tokyo, Japan), which enabled separation of CM-R and VLDL remnants from other lipoproteins with higher specificity than the remnant like particle-cholesterol method [12,13]. Before ezetimibe administration, RemL-C and apoB-48 levels were higher in enrolled patients than in normolipidaemic subjects, in conjunction with higher levels of TC, TG, apoB and LDL-C (patients vs. normolipidemic subjects shown in the previous studies: RemL-C 18·7 ± 10·5 vs. 3·5 ± 1·2 mg dL^{-1} in [13]; apoB-48 6·8 ± 4·3 vs. 5·2 ± 3·8 μg mL^{-1} in [11]).

Oral fat loading test

The OFL test was performed before and after the administration of ezetimibe. After an overnight fast for 12 h, oral fat tolerance test (OFTT) cream which was prepared from milk and adjusted to contain 35% fat without sugar (JOMO Foods, Gunma, Japan) was loaded to each patient sufficient to provide a fat load of 30 g fat m^{-2} body surface area. Blood samples were drawn before and 1, 2, 3, 4, 6 and 8 h after OFL and concentrations of TC, TG, apo B-48, FFA, RemL-C and apoB-100 were measured. To compare the net postprandial change in

these parameters, areas under the curve (AUC) for TC, TG, apo B-48, FFA, RemL-C and apoB-100 were calculated using the trapezoidal method and incremental AUC (ΔAUC) values by ignoring area beneath the fasting level.

Lipoprotein profiles assessed by HPLC

The effect of ezetimibe on lipoprotein profile during fasting and 4 h after OFL was evaluated using the HPLC method. Samples of 200 microlitres of serum (fasting state and 4 h after OFL before and after administration of ezetimibe) were analysed at Skylight Biotech Inc. (Akita, Japan) and dissolved with the loading buffer (0·05 mol L^{-1} Tris-buffered acetate, pH 8·0). These samples were loaded into two tandem connected TSK-gel Lipopropak XL columns and concentrations of TC and TG in the flow-through of each sample were measured continuously and simultaneously [14]. The flow-through of dissolved serum (n = 10) which was drawn 4 h after OFL was collected serially every 1 min into collection tubes (tube No. 1-20) both before and after administration of ezetimibe. The apoB-48 levels of tube No. 1-11 which were supposed to contain lipoproteins in the size range of CM (tube No.1-2), VLDL (tube No. 3-7) and LDL (tube No. 8-10) were measured using the method as mentioned above. The beginning and ending time of the collection of the flow-through was shown in the chromatographic pattern using grey bars in Fig. 3a. We calculated cholesterol and TG concentrations of lipoprotein fractions in the size categories of CM, VLDL, LDL and HDL, based on findings from a prior investigation that confirmed the correspondence of lipoprotein fractions in CM, VLDL, LDL, and HDL-sizes and the elution time, by comparing the HPLC pattern of each lipoprotein separated using ultracentrifugation [15]. Those categories were as follows: CM-size, estimated particle size > 80 nm, elution time 15–17 min; VLDL size 30–80 nm, 17–22 min; LDL size, 16–30 nm, 22–25·5 min; HDL-size, 8–16 nm, 25·5–28·5 min.

Statistical analyses

The results were expressed as mean ± SD. The Student's paired t-test was used for pairwise comparisons between values before and after administration of ezetimibe. A value of $P < 0·05$ was considered to be statistically significant.

Results

Effect of ezetimibe on fasting serum levels of lipid biomarkers in patients with type IIb hyperlipidaemia

Table 1 shows fasting serum levels of lipid biomarkers before and after administration of ezetimibe for 2 months. Ezetimibe effectively reduced serum levels of TC, TG, apoB and LDL-C in the fasting state as we expected. LDL-C reducing response varied between 9·8% (reducing from 151 to 136 mg dL^{-1}) and 33·2% (from 152 to 101 mg dL^{-1}). However, the mean rate of reduction in TG was larger than previously reported for ezetimibe treatment in patients with primary hypercholesterolaemia (mean reduction rates: TC -16·5%, TG -24·5%, apoB -15·7, LDL-C -20·3%). It is especially striking that fasting levels of apoB-48, and RemL-C were also significantly decreased after the administration of ezetimibe (mean reduction rates: RemL-C -22%, apoB-48 -31%) in type IIb hyperlipidaemic patients. These results suggest that ezetimibe may affect not only VLDL and LDL particles containing apoB-100, but also CM and CM-R particles containing apoB-48. There was no difference in body weight and waist circumference through the treatment. Ezetimibe treatment did not alter serum levels of HDL cholesterol, apoAI, apoAII, apoCIII, apoE, FFA and diabetic parameters, fasting plasma glucose, plasma insulin or haemoglobin A1c

Table 1 Fasting levels of lipid biomarkers before and after administration of ezetimibe

		Ezetimibe(−)	Ezetimibe(+)	*P*-value
TC	(mg dL^{-1})	231 ± 43	194 ± 26	0·001
TG	(mg dL^{-1})	218 ± 83	178 ± 85	0·031
LDL-C	(mg dL^{-1})	145 ± 42	120 ± 25	0·005
HDL-C	(mg dL^{-1})	53 ± 14	52 ± 13	0·394
FFA	(μEq L^{-1})	508 ± 187	483 ± 184	0·270
RemL-C	(mg dL^{-1})	18·7 ± 10·5	12·0 ± 6·3	0·006
apoAI	(mg dL^{-1})	144 ± 29	142 ± 31	0·130
apoAII	(mg dL^{-1})	32·2 ± 8·0	30·8 ± 7·6	0·071
apoB-100	(mg dL^{-1})	116 ± 22	101 ± 13	0·004
apoB-48	(μg mL^{-1})	6·8 ± 4·3	4·7 ± 2·3	0·019
apoCII	(mg dL^{-1})	5·3 ± 2·8	4·3 ± 2·1	0·043
apoCIII	(mg dL^{-1})	11·7 ± 4·3	10·5 ± 3·8	0·082
apoE	(mg dL^{-1})	6·2 ± 1·3	5·6 ± 1·4	0·054
Glucose	(mg dL^{-1})	107 ± 21	104 ± 19	0·165
Insulin	(μIU mL^{-1})	12·1 ± 5·5	14·5 ± 5·5	0·231
HOMA-IR		3·2 ± 1·6	3·7 ± 3·6	0·165
HbA1c	(%)	5·6 ± 0·4	5·5 ± 0·4	0·165
hs-CRP	mg dL^{-1}	0·11 ± 0·08	0·16 ± 0·15	0·17

TC, total cholesterol; TG, triglyceride; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; FFA, free fatty acid; RemL-C; remnant lipoprotein cholesterol; apo, apolipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; HbA1c, haemoglobin A1c; hs-CRP; high sensitivity C reactive protein.
HOMA-IR index was calculated as [fasting plasma insulin (μ IU mL^{-1}) × fasting plasma glucose (mg dL^{-1})]/405.
Data were shown as mean ± SD and statistical significance was calculated using paired t-test.

levels as well as HOMA-IR index (Table 1). In this study, there was no significant change in the levels of hs-CRP which is an independent marker for the development of atherosclerotic cardiovascular diseases by ezetimibe treatment.

Effect of ezetimibe on fasting lipoprotein profiles in patients with type IIb hyperlipidaemia

To evaluate the effect of ezetimibe on fasting lipoprotein profiles, serum samples were analysed by HPLC, and cholesterol and TG levels were measured. Representative chromatographic patterns of cholesterol and TG before and after ezetimibe treatment are shown in Fig. 1a. For each patient cholesterol and TG levels in the indicated pooled fractions corresponding to CM-, VLDL-, LDL- or HDL-sized particles were summed and averages were calculated. The levels of cholesterol and TG decreased in the VLDL and LDL fractions after ezetimibe treatment, and the LDL peak in cholesterol tended to shift slightly to the left (lower elution time, greater apparent size), which may represent large LDL particles (before vs. after administration of ezetimibe: VLDL-C 46 ± 13 vs. 32 ± 12 mg dL^{-1}, $P = 0{\cdot}0016$; LDL-C 150 ± 33 vs. 120 ± 27, $P = 0{\cdot}0018$; VLDL-TG 176 ± 67 vs. 116 ± 54, $P = 0{\cdot}0027$; LDL-TG 49 ± 12 vs. 41 ± 7, $P = 0{\cdot}034$). However, this shift was not observed in all specimens. Findings for cholesterol and TG content in CM- and HDL-size particles after ezetimibe treatment were similar to the treatment baseline.

OFL test before and after ezetimibe administration

As shown in Fig. 2, postprandial changes in lipid profiles were determined by OFL testing with OFTT cream before and after administration of ezetimibe in 10 patients with type IIb hyperlipidaemia. Initial values for serum TC and apoB-100 after

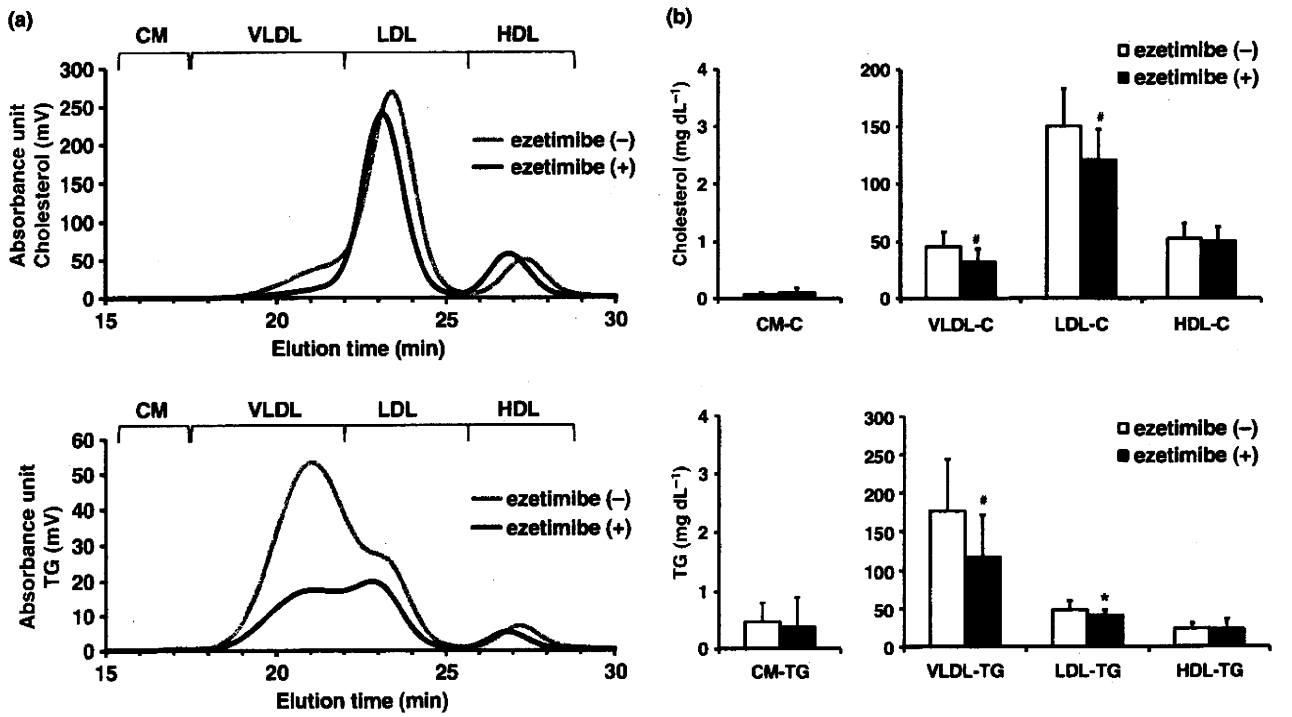

Figure 1 Lipoprotein profiles in the fasting state before and after administration of ezetimibe. Ezetimibe 10 mg was administered in patients with type IIb hyperlipidaemia (n = 10, two females and eight males) for 2 months. Two hundred microlitres of serum were separated from blood drawn in the fasting state before and after administration of ezetimibe. Lipoprotein profiles were analysed by high performance liquid chromatography. The concentrations of cholesterol and triglyceride (TG) in the flow-through of each sample were measured continuously and simultaneously. (a) Representative chromatograms of cholesterol and TG of fasting serum before (grey line) and after (black line) administration of ezetimibe were shown with approximate elution times of chylomicrons (CM), very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). (b) For each patient cholesterol and TG levels in the indicated pooled fractions corresponding to CM-, VLDL-, LDL- or HDL-size particles were summed and averages were calculated before (open squares) and after (closed squares) administration of ezetimibe. *$P < 0{\cdot}05$, #$P < 0{\cdot}005$.

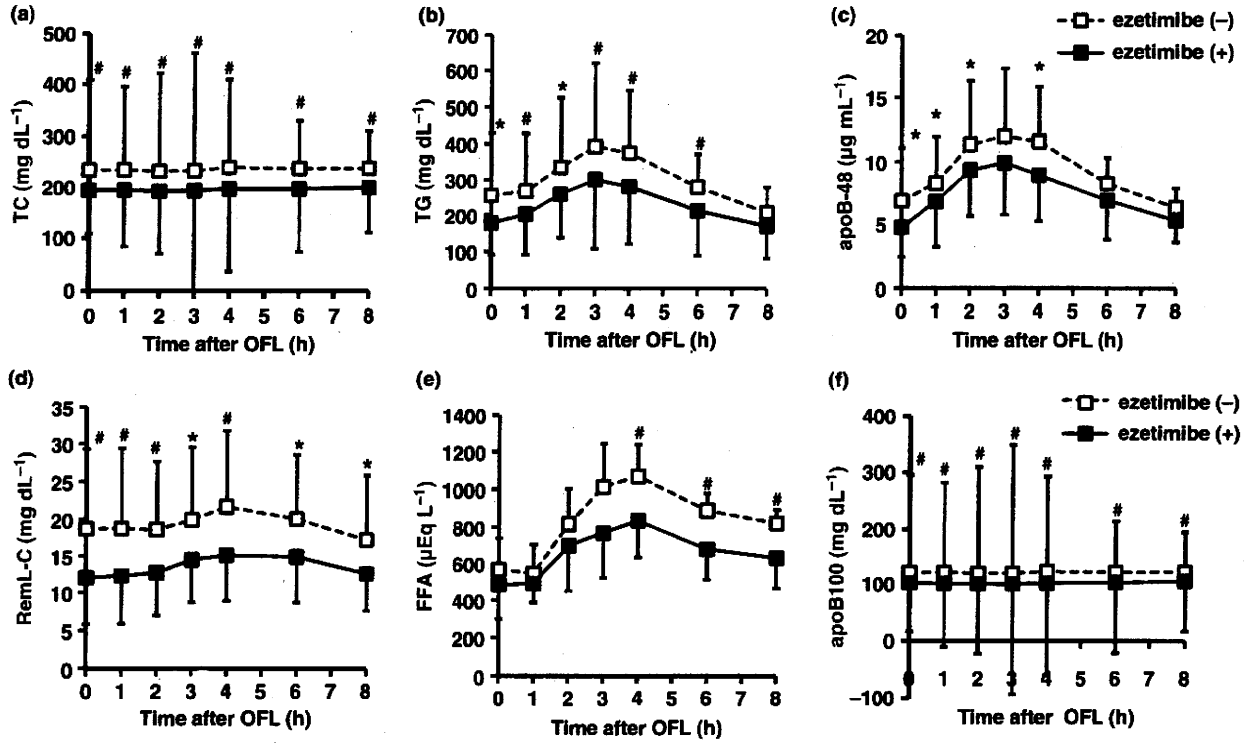

Figure 2 Oral fat loading (OFL) test before and after administration of ezetimibe. Patients with type IIb hyperlipidaemia (n = 10, two females and eight males) were given OFTT cream (containing 35% fat without sugar, 30 g fat m^{-2} body surface area) after overnight fasting before (open squares) and after (closed squares) administration of ezetimibe. Blood samples were drawn during fasting and 1, 2, 3, 4, 6 and 8 h after OFL, and serum and plasma were separated immediately. Concentrations of (a) total cholesterol (TC), (b) triglyceride (TG), (c) apolipoprotein B-48(apoB-48), (d) remnant lipoprotein cholesterol (RemL-C), (e) free fatty acids (FFA) and (f) apoB-100 were measured as described in Materials and methods. *P < 0·05, #P < 0·01.

ezetimibe treatment were significantly lower than before the treatment. Serum TC and apoB-100 levels remained constant throughout the 8-h OFL test. TG, apoB-48 and RemL-C levels rose for the first 3 or 4 h, and returned to fasting levels 8 h after OFL. Ezetimibe significantly diminished fasting and peak levels for these parameters and for AUC, which reflects the postprandial integrated response (AUC-TC 1892 ± 350 vs. 1570 ± 204 mg dL^{-1} 8 h, P = 0·0001; AUC-apoB-100 2167 ± 649 vs. 1519 ± 488 mg dL^{-1} 8 h, P = 0·023; AUC-TG 2448 ± 1130 vs. 1863 ± 1012 mg dL^{-1} 8 h, P = 0·003; AUC-apoB-48 75 ± 23 vs. 61 ± 22 μg dL^{-1} 8 h, P = 0·044; AUC-RemL-C 156 ± 72 vs. 110 ± 46 mg dL^{-1} 8 h, P = 0·008). However, incremental AUCs (ΔAUCs), which are thought to describe postprandial integrated response more accurately, after ezetimibe administration were comparable to the corresponding values before ezetimibe administration for TG, apoB-48 and RemL-C (ΔAUC-TC 11 ± 98 vs. 15 ± 61 mg dL^{-1} 8 h, P = 0·448; ΔAUC-apoB-100 483 ± 334 vs. 236 ± 318 mg dL^{-1} 8 h, P = 0·168; ΔAUC-TG 405 ± 442 vs. 443 ± 553 mg dL^{-1} 8 h, P = 0·442; ΔAUC-apoB-48 21 ± 33 vs. 23 ± 17 μg dL^{-1} 8 h, P = 0·394; ΔAUC-RemL-C 6·5 ± 22 vs. 14 ± 14 mg dL^{-1} 8 h, P = 0·432). Ezetimibe intervention reduced peak level, AUC and ΔAUC for FFA after OFL (AUC-FFA 6856 ± 1362 vs. 5433 ± 1231 mg dL^{-1} 8 h, P = 0·004; ΔAUC-FFA, 2329 ± 1159 vs. 1564 ± 1249 mg dL^{-1} 8 h, P = 0·017), indicating a possible decrease in FFA production and/or increase in FFA clearance. There were no changes in serum levels for other apolipoproteins (apoAI, AII, CII, CIII, and E) throughout the OFL test, either before or after ezetimibe treatment (data not shown).

HPLC analysis of postprandial lipoprotein profiles

To further elaborate on postprandial lipid changes, HPLC analysis was conducted 4 h after the OFL test to compare cholesterol and TG concentrations of lipoprotein fractions in the CM, VLDL, LDL and HDL-size ranges before and after administration of ezetimibe. Chromatographic patterns of

serum 4 h after OFL revealed that three peaks were observed in the size range of CM, VLDL and LDL by the detection of cholesterol and TG levels. The VLDL peak by the detection of TG after OFL was shifted to the left (lower elution time, greater apparent size) compared with that in the fasting state, suggesting that any other lipoprotein particles which were contained in the size range of VLDL and larger than VLDL observed in the fasting state were produced after OFL. HPLC analysis of serum which was obtained 4 h after OFL before and after ezetimibe treatment showed that three peaks by the detection of TG in the size range of CM, VLDL and LDL tended to decrease after ezetimibe treatment (Fig. 3a). By the calculation of average cholesterol and TG levels in the size range of CM, VLDL, LDL and HDL, HPLC analysis 4 h after OFL revealed that the reduction in serum TC and TG after ezetimibe treatment was mainly due to cholesterol and TG changes in the size range of CM and VLDL, not due to those in the size range of LDL (CM-C 0·63 ± 0·26 vs. 0·31 ± 0·09 mg dL^{-1}, $P = 0·0029$; VLDL-C 50 ± 14 vs. 37 ± 11 mg dL^{-1}, $P = 0·0022$; LDL-C 138 ± 41 vs. 116 ± 2 mg dL^{-1}, $P = 0·059$; CM-TG 10·2 ± 5·4 vs. 4·7 ± 2·2 mg dL^{-1}, $P = 0·014$; VLDL-TG 251 ± 93 vs. 180 ± 88 mg dL^{-1}, $P = 0·0009$, LDL-TG 50 ± 13 vs. 43 ± 8 mg dL^{-1}, $P = 0·056$) (Fig. 3b). Furthermore, to evaluate whether CM-R were contained in the size range of VLDL and LDL 4 h after OFL and their contents were changed before and after ezetimibe treatment, we measured apoB-48 levels of serially collected flow-through of dissolved serum ($n = 10$) which was drawn 4 h after OFL, as shown in Materials and methods (Fig. 3a). Both before and after ezetimibe treatment, apoB-48 was detected in the fractioned flow-through which was suggested to contain lipoproteins in the size range of not only CM but also VLDL and LDL (Fig. 1a). Before ezetimibe treatment, we can see two peaks of apoB-48 levels at the position of tube No. 5 and No. 8, which was coincided with peaks by the detection of TG in the size range of VLDL and LDL 4 after OFL. These findings suggested that CM-R particles existed in various size ranges, from the size of CM to HDL, and the peak of the size of CM-R particles existed both in the size range of VLDL and LDL. After ezetimibe treatment, apoB-48 levels were decreased in all size ranges and the peak of apoB-48 levels in the size range of VLDL had disappeared. However, the decreases in apoB-48 levels by ezetimibe treatment were significant in tube No.1, No. 7 and No. 8, but not significant in other tubes. (No.1:before vs after treatment, 0·012 ± 0·008 vs. 0·003 ± 0·001 μg dL^{-1}, $P = 0·020$, No. 7; 0·031 ± 0·020 vs. 0·013 ± 0·003 μg dL^{-1}, $P = 0·043$, No.8; 0·044 ± 0·018 vs. 0·018 ± 0·006 μg dL^{-1}, $P = 0·021$). These results suggested that the decreases in particle numbers of CM and CM-R by the ezetimibe treatment occurred significantly in the size range of CM and small VLDL, but relatively in the size range of large VLDL particles. To address whether suppression of lipoprotein production resulted in any reduction of TG and cholesterol in the size range of CM and VLDL particles after ezetimibe administration, we calculated differences in cholesterol and TG levels in the size range of CM and VLDL particles between fasting and 4-h OFL, and compared these differences before and after ezetimibe treatment (Fig. 3c). Ezetimibe attenuated the increase in cholesterol level at the CM-size (0·56 ± 0·25 vs. 0·21 ± 0·11 mg dL^{-1}, $P = 0·0008$), which might reflect the inhibition of cholesterol absorption in the intestine in accordance with the mechanism of action of ezetimibe. In a particularly striking finding, the increase in CM-size TG was also attenuated after the administration of ezetimibe (9·7 ± 5·4 vs. 4·4 ± 2·3 mg dL^{-1}, $P = 0·017$) (Fig. 3c) along with the significant decrease in apoB-48 level in the size range of CM 4 h after OFL (Fig. 3a), which raised the possibility that the decreased intestinal cholesterol absorption associated with ezetimibe administration might also influence the intestinal production of CM. There were no significant differences before and after ezetimibe treatment in increased VLDL particle size levels for cholesterol or TG between fasting and 4-h OFL, even though ezetimibe decreased the fasting and postprandial (4 h after initiating OFL test) TG levels for the VLDL size range (Figs 1 and 3). These findings suggest that VLDL metabolism, at least during the 4-h OFL test, was unaffected by ezetimibe treatment.

Discussion

In this study, we elucidated the fasting and postprandial lipid and lipoprotein profiles of patients with type IIb hyperlipidaemia before and after ezetimibe administration. We clearly showed that ezetimibe treatment decreased the fasting apoB-48 and RemL-C levels as well as TC, TG, and apoB-100 levels. When we subtracted apoB-48 levels from the apoB levels, the resulting values also showed decreases in apoB-100 after ezetimibe administration. HPLC analysis showed reduced levels of cholesterol and TG in VLDL and LDL fractions at fasting after ezetimibe administration, suggesting that the levels of apoB-100-containing lipoproteins such as VLDL, VLDL remnants and LDL particles were reduced in conjunction with decreased serum apoB-100 levels. Telford *et al.* demonstrated, in a study of miniature pigs, that ezetimibe decreased the intrahepatic cholesterol pool through inhibition of intestinal cholesterol absorption, leading to the suppression of hepatic VLDL production and enhanced LDL clearance by upregulation of LDL receptor expression in hepatocytes [16]. As a consequence, serum levels of apoB-100-containing lipoproteins were reportedly reduced after ezetimibe administration in that experiment. Unlike rodents, humans express high levels of NPC1L1 protein in the liver as well as in the intestine. A study using liver-specific NPC1L1 transgenic mice has indicated that the function of liver NPC1L1 was to take up cholesterol from

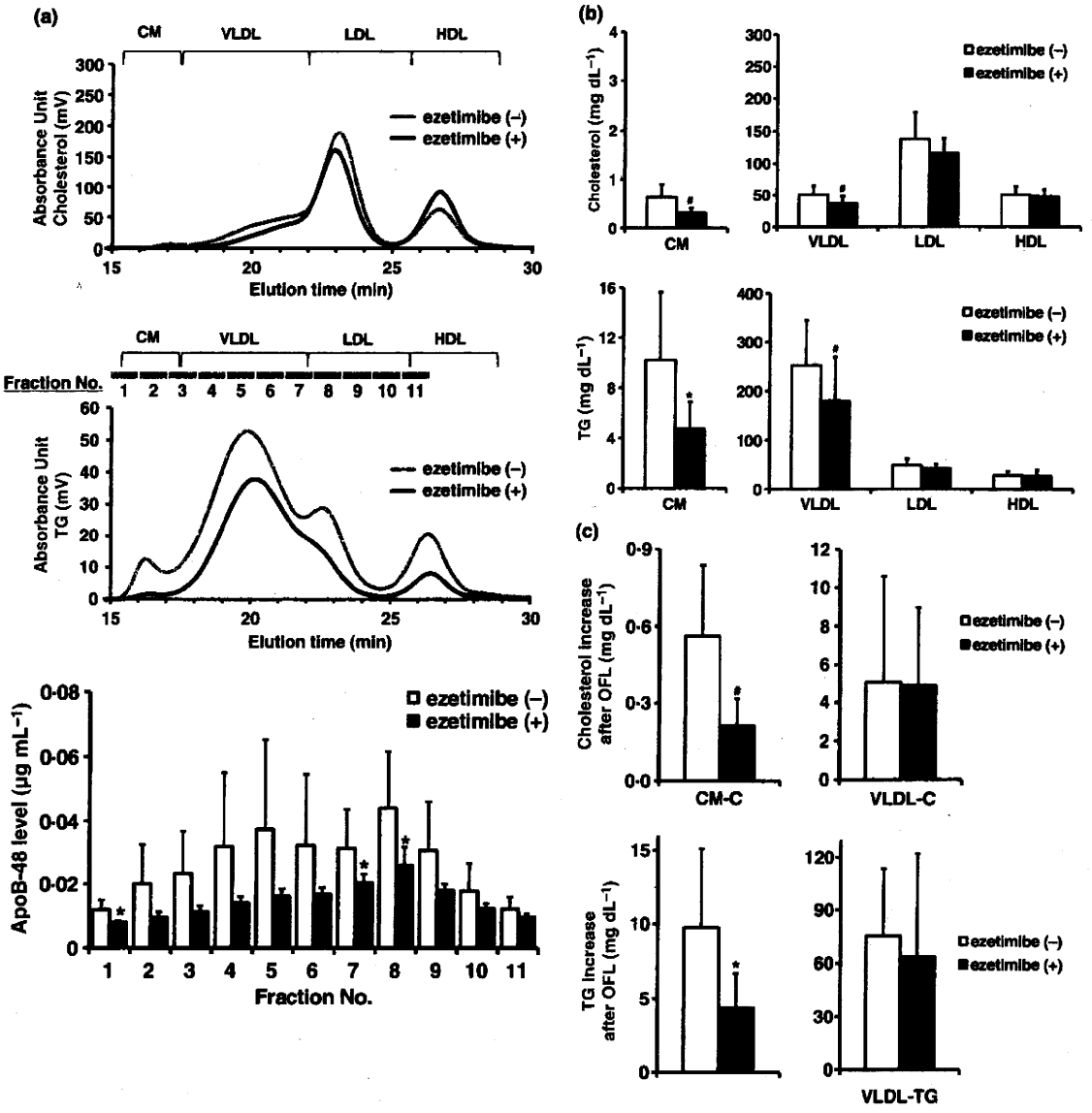

Figure 3 Lipoprotein profiles in postprandial state and incremental fasting/postprandial serum cholesterol and triglyceride (TG) levels before and after administration of ezetimibe. Two hundred microlitres of serum were separated from blood samples drawn 4 h after oral fat loading (OFL) before (open squares) and after (closed squares) administration of ezetimibe for 2 months in patients with type IIb hyperlipidaemia (n = 10, two females and eight males). (a) Representative chromatograms of cholesterol and TG of serum 4 h after the OFL before (grey line) and after (black line) administration of ezetimibe are shown with approximate elution times of chylomicrons (CM), very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). The flow-through of dissolved serum was collected serially every 1 min into collection tubes (tube No. 1-20), apoB-48 levels of tube No. 1-11 which were supposed to contain lipoprotein in the size range of CM, VLDL and LDL were measured using a chemiluminescent enzyme immunoassay method. Grey bars indicate the beginning and ending time of the collection of the flow-through. (b) For each patient (n = 10), cholesterol and TG concentrations of lipoprotein fractions in the size range of CM, VLDL, LDL and HDL were calculated before (open squares) and after (closed squares) administration of ezetimibe. (c) Incremental serum cholesterol and TG levels in the indicated pooled fractions corresponding to CM- or VLDL size particles between fasting and postprandial (4 h after OFL) states were calculated before (open squares) and after (closed squares) ezetimibe treatment. *P < 0·05, #P < 0·005.

bile acids and return it to the liver, and that hepatic NPC1L1 was also targeted by ezetimibe [17]. Inhibition of hepatic NPC1L1 by ezetimibe would result in attenuation of the hepatic cholesterol pool caused by a relative increase of cholesterol secretion into bile acids. It would be appropriate to contextualize these reports, at least partially, by explaining the mechanism that we detected, whereby apoB-100-containing lipoprotein levels were diminished at fasting after ezetimibe treatment. ApoB-48 incorporated into CM and CM-R was also reduced at fasting, whereas cholesterol and TG contents in the CM fraction were unaltered on HPLC analysis. In our previous study, we reported that apoB-48 protein was also detected by western blotting, in the flow-through analytes for elution time between 19 and 22 min which overlapped the VLDL fraction. In this study, we were able to detect apoB-48 in these subfractions by a CLEIA method. Those findings proved that the lipoprotein fraction in the VLDL size range contained CM-R as well [18] in the HPLC system that we used. This can explain our findings of apoB-48 reduction with no alteration of cholesterol or TG content in the CM-size fraction, and taken together with the findings from this study, demonstrates that ezetimibe treatment decreased the level of fasting CM-R. Similar to the ezetimibe-induced modification of the metabolism of apoB-100-containing lipoproteins, the inhibition of cholesterol inflow into the liver might cause upregulation of remnant receptors, which would improve clearance of CM-R and reduce serum CM-R levels. As the fasting levels of CM-size particles remained unchanged after ezetimibe treatment, there are some remaining issues to be addressed regarding whether ezetimibe may facilitate LPL activity, although we did not measure LPL activity in this study.

These discoveries are relevant to the alteration of fasting TRL serum levels, and imply that ezetimibe might modify postprandial TRL metabolism as well, as sustained accumulation of TRL particles in the blood after a meal induces high fasting levels for TRL. Our results from the OFL test conducted in patients with type IIb hyperlipidaemia supported this hypothesis. It is well documented that TC, apoB-100 and LDL-cholesterol levels are unaffected by the OFL test under normal conditions. Consistent with the previous observation, serum TC and apoB-100 levels showed constant values throughout the 8-h OFL test both before and after ezetimibe treatment, although initial values for serum TC after administration were significantly lower than before administration, reflecting decreased fasting TC and apoB-100 levels. Ezetimibe intervention significantly diminished fasting and peak levels for TG, RemL-C and apoB-48, and those respective AUC values in the OFL test, whereas the corresponding ΔAUCs were comparable to those values before ezetimibe treatment. These findings suggest that very few additional effects other than reduction of initial levels were observed in this experiment. However, further detailed examination of lipoprotein profiles by HPLC, performed 4 h after the OFL test, revealed striking evidence that ezetimibe did incrementally attenuate both cholesterol and TG levels with regard to the size of CM but not the size of VLDL particles. Especially, we measured the apoB-48 levels of serially collected flow-through of dissolved serum which was drawn 4 h after OFL and evaluated changes of CM-R particles by ezetimibe treatment in the size range of CM, VLDL and LDL (Fig. 3a). As a result, CM-R particles existed in various size ranges, from the size of CM to HDL and their peak existed both in the size range of VLDL and LDL. After ezetimibe treatment, apoB-48 levels were decreased in all size ranges and the peak of apoB-48 levels in the size range of VLDL disappeared. However, the decreases in apoB-48 levels by ezetimibe treatment were significant only in the size range of CM and small VLDL, but not in the size range of large VLDL (Fig. 3a). These results suggested that the decreases in particle numbers of CM and CM-R by the ezetimibe treatment occurred significantly in the size range of CM and small VLDL, but relatively in the size range of large VLDL particles. It can be speculated that, because both production of CM and catabolism of CM and CM-R may be accelerated with ezetimibe treatment, the reduction of CM was apparent based upon the reduction of apoB-48 levels in CM-size range; however, the reduction of CM-R in the size range of VLDL was not apparent. This suggests the possibility that intestinal CM production was reduced significantly and CM-R which were in the size range of VLDL and LDL were relatively decreased, but hepatic VLDL production was unaffected by ezetimibe during the 4-h OFL as there was no increase in apoB-100 levels by the OFL both with and without ezetimibe treatment. These changes in lipoprotein profiles were substantial, and the effect of ezetimibe on postprandial TLR metabolism could be underestimated if those changes were disregarded. As we did not measure LPL activity or compounds like retinyl palmitate, we could not deny the possibility for the improvement of the impaired catabolism of CM and CM-R.

The only parameter showing reduced ΔAUC after ezetimibe administration was FFA. Recently Labonte ED *et al.* reported that ezetimibe-treated mice absorbed only 86·9% of the fat from a high-fat, high-sucrose diet compared with 94·9% of fat absorption in untreated mice [19]. Our loading fat, OFTT cream, contains 35% fat and has a main fatty acid composition of C16:0, C18:1 and C14:0. According to the Labonte experiments, absorption of palmitate, oleate and myristate was decreased from 89·0, 95·9 and 93·5% in the controls to 79·2, 91·2, and 87·7% respectively in ezetimibe-treated mice. In addition, there was a 50% reduction in expression of FATP4 protein in intestinal preparations from ezetimibe-treated mice in comparison with the control mice and a 35% reduction in CD36 protein expression. Both of these proteins are considered to play

important roles in FFA transport. These observations might apply under our experimental conditions as well, although mice received chronic exposure to a high-fat and high-sucrose diet and FFA measurements were fasting values. Once FFA are absorbed by the enterocytes, it is used for the resynthesis of TG, along with monoacylglycerols that are believed to be absorbed by passive diffusion from the gut lumen. TG is incorporated into CM and released into the thoracic lymph, a process that involves many molecules related to the assembly and secretion of CM. Next, CM passes into the bloodstream and is exposed to LPL, resulting in the discharge of FFA from CM to serum. We could speculate that decreased FFA absorption after ezetimibe treatment in acute fat loading led to the reduction in ΔAUC for FFA. FFA are also taken up in the adipose tissue for energy storage and in striated muscles for combustion. This might be less likely to play a role in the ezetimibe-induced reduction of postprandial ΔAUC for FFA, as there were no changes in patient body weight or waist circumference during the 2 months of the study and as fasting FFA levels also remained unchanged. The reduction in postprandial intestinal CM production that was associated with ezetimibe treatment could be a consequence of chronic cholesterol shortage and reduced FFA absorption in the intestinal epithelium.

Our findings in this study suggested some treatment options for patients with combined hyperlipidaemia. There has been an ongoing argument regarding whether patients with type IIb hyperlipidaemia should be treated with statins for hypercholesterolaemia or fibrates for hypertriglyceridaemia. (Dual therapy is not an attractive option, as the combined use of statins and fibrates is associated with a higher frequency of the severe life-threatening side effect of rhabdomyolysis.). In this study, the administration of ezetimibe improved endogenous and exogenous TRL profiles by suppressing postprandial intestinal production of CM and possibly by reducing the fasting hepatic cholesterol pool. Ezetimibe administration can thus be a favourable option for the treatment of patients with elevated VLDL, LDL and remnant lipoproteins. Several studies have shown that ezetimibe improved lipid metabolism in obese patients with dyslipidaemia and in animal models for metabolic syndrome [20–22] and one of those studies also showed a concomitant improvement in insulin response. Moreover, ezetimibe has been reported to inhibit elevation of hs-CRP [23] and to improve endothelium-dependent acetylcholine-induced vasodilatation in patients with metabolic syndrome [24]. There thus appear to be numerous pleiotrophic effects of ezetimibe on ameliorating cardiovascular risk factors. More evidence from mega-trials can be expected to clarify the anti-atherogenic effects of ezetimibe in cardiovascular disease accompanied by accumulation of remnant lipoproteins.

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Original Article

Fenofibrate Reduces Postprandial Hypertriglyceridemia in CD36 Knockout Mice

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Aim: Metabolic syndrome (MetS) and postprandial hypertriglyceridemia (PHTG) are closely related and both are associated with coronary heart disease. We have demonstrated that CD36 deficiency is prevalent in the genetic background of MetS and is accompanied by PHTG concomitantly with an increase in remnants and a decrease in high density lipoprotein cholesterol. These findings make CD36 knockout mice (CD36KO) an interesting model for evaluating PHTG in MetS. Fenofibrate was reported to reduce fasting and postprandial triglyceride (TG) levels in hypertriglyceridemic subjects with MetS. To define its mechanism, we investigated the effect of fenofibrate on PHTG in CD36KO.

Methods: Wild-type (WT) and CD36KO mice were fed chow diet and fenofibrate for two weeks. TG concentrations and lipoprotein profiles were assessed during fasting and in the postprandial state in plasma; intestinal mucosa and lymph were collected after oral fat loading for both treatment groups.

Results: Fenofibrate treatment markedly suppressed the postprandial TG response in CD36KO along with decreased apoB-48 levels in plasma. HPLC analysis depicted the decrease of TG content in chylomicrons (CM) and CM remnant-sized lipoproteins contributed to this suppression, suggesting that CM and CM remnant production in the intestines might be attenuated by fenofibrate. ApoB-48 and TG levels in intestinal lymph were markedly reduced after treatment. Intestinal mRNA expression of apoB was also reduced in the postprandial state after fenofibrate administration without affecting any other genes related to CM assembly and production.

Conclusion: Fenofibrate reduces PHTG in CD36KO partially through attenuating intestinal CM production.

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Key words; Fenofibrate, Postprandial hypertriglyceridemia, CD36 knockout mice, Apolipoprotein B-48

Introduction

Metabolic syndrome (MetS), based upon the accumulation of visceral fat, represents a clustering of interrelated risk factors for cardiovascular disease that include abnormally high serum triglyceride (TG) levels in the fasting state[1, 2]. Metabolic syndrome presents as a challenge to the healthcare system, particularly due to the increasing prevalence of overweight/obesity and type 2 diabetes mellitus worldwide[3].

The publication of meta-analyses pointing at raised serum TG levels as an independent risk factor for coronary heart disease highly suggests that TG-rich lipoproteins, such as chylomicrons (CM), very low

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density lipoproteins (VLDL) and their remnants, are atherogenic[4, 5]. Triglycerides are routinely measured in the fasting state, excluding CM and their remnants; however, elevated non-fasting TG levels were found to be associated with an increased risk of coronary artery disease, stroke and death in men and women[6-8], which suggests atherosclerosis as a postprandial phenomenon where CM and CM remnants would play an important role. Thus, increased levels of non-fasting TG, as well as increased levels of CM and CM remnants, should constitute a potentially important predictor of atherosclerotic cardiovascular diseases, and the strong evidence supporting the independent atherogenicity of these remnants[9] makes them appropriate targets for lipid-lowering therapy.

CD36, also known as fatty acid (FA) translocase, an 88 kD glycoprotein belonging to the scavenger receptor class B, has been shown to bind multiple ligands, including long-chain FAs and oxidized low density lipoproteins[10]. CD36 is broadly expressed in many cells, such as monocytes, platelets, macrophages, microvascular endothelial cells, adipocytes, skeletal and cardiac myocytes, enterocytes and Kupffer cells[11]. Human CD36 deficiency is accompanied by multiple risk factors, such as increased remnant lipoproteins and low high density lipoproteins (HDL) cholesterol, as well as impaired glucose metabolism, based upon insulin resistance. These findings suggested that this condition may be considered a genetic background for MetS[12, 13]. CD36 knockout (CD36KO) mice have been also demonstrated to increase the postprandial plasma TG and FA response after an acute oral fat loading of more than 2-fold compared to wild-type (WT) mice[14]. We demonstrated a postprandial increase of plasma CM-remnants with enhanced TG synthesis in the small intestine of CD36KO compared to WT mice and suggested that the main cause for the postprandial elevation of TG in plasma was the *de novo* synthesis of small-sized CM in enterocytes[15]. These findings strongly suggest CD36KO mice as an interesting model to evaluate postprandial hypertriglyceridemia in a MetS environment.

Peroxisome proliferator activated receptor (PPAR) alpha is a ligand-activated transcription factor with diverse functions, expressed in a variety of tissues[16], and is activated by several synthetic compounds. Fenofibrate, a PPAR-α ligand, has been demonstrated to reduce TG levels in fasting and postprandial states in a cohort of hypertriglyceridemic subjects with MetS; this TG-lowering effect resulted primarily from reductions in fasting and postprandial concentrations of large and medium VLDL particles[17]. Moreover, fenofibrate has been shown to reduce non-fatal myocardial infarctions and coronary revascularizations in diabetic patients[18].

To elucidate the effect of fenofibrate on postprandial hypertriglyceridemia in CD36KO mice, we performed an oral fat-loading test before and after fenofibrate treatment and demonstrated that fenofibrate reduced postprandial hypertriglyceridemia, thus promoting a protective effect against atherosclerosis in a mouse model for MetS.

Materials and Methods

Animals

Male CD36KO mice created on a C57BL6/J background, which were kindly provided by Mason. W. Freeman, M.D., Ph.D., Professor of Harvard Medical School[19], and C57BL6/J WT mice at 8-10 weeks of age were used for this experiment. Each strain of mice was separated into two groups, which were fed chow diet (MF, Oriental BioLaboratories, Chiba, Japan) alone or chow diet containing 0.05% fenofibrate (Aska Pharmaceuticals, Tokyo, Japan) for 2 weeks. The mice were housed in a temperature-controlled environment with a 12-hour dark-light cycle and free access to food and water. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine (IEXAS). After 2 weeks of treatment, each strain was fasted for 12 hours and separated into two groups to be euthanized: in the fasting state and three hours after acute ingestion of 17 μL/g body weight of olive oil (Nacalai Tesque, Kyoto, Japan) by gavaging.

Triglyceride Determination and Lipoprotein Analysis of Plasma and Intestinal Lymph

Plasma and lymph TG concentrations were measured enzymatically (Wako Pure Chemical Industries, Tokyo, Japan) according to the manufacturer's protocol.

The plasma and lymph lipoprotein profile was analyzed by an online dual enzymatic method using high performance liquid chromatography (HPLC) at Skylight Biotech Inc. (Akita, Japan)[20], where 200 μL of plasma or lymph were dissolved in loading buffer and loaded onto TSK gel Lipopropak XL columns. Triglyceride concentrations in the flow-through were measured continuously and simultaneously. The correspondence of lipoprotein fractions (CM, VLDL, LDL, and HDL-sized fractions) and the elution time were CM (>80 nm, fraction time: 15-17 min), VLDL (30-80 nm, fraction time: 17-22 min), LDL (16-30 nm, fraction time: 22-28 min), and HDL (8-16 nm,

fraction time: 28–37 min).

Collection of Intestinal Lymph in the Postprandial State

Five mice from each strain were loaded with olive oil (17 μL/g body weight) after a fasting period of 12 hours. Three hours later, mice were anesthetized and the intestinal lymphatic trunk was cannulated using a 27-gauge needle inserted into a polyethylene tube (PE-50) previously flushed with EDTA-treated water, according to the modified method described by Bollman *et al.*[21].

Western Blot

One microliter of plasma or lymph was subjected to 4–12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE; TEFCO, Tokyo, Japan), transferred onto an Immobilon-P transfer membrane (Millipore Corp., USA) and blocked by Blocking One (Nacalai Tesque, Kyoto, Japan). The membrane was then incubated with anti-mouse apoB-48/B-100 antibody (BIODESIGN International, ME, USA) and anti-rabbit IgG antibody (NA934V; GE Healthcare Backinghamshire, UK). Bands corresponding to apoB-100 and apoB-48 were detected with the ECL Advance Detection Kit (GE Healthcare, UK).

RNA Extraction, cDNA Synthesis and Quantitative Real-Time PCR

The small intestine from each animal was removed, flushed with ice-cold phosphate-buffered saline and divided into three sections of equal length, the proximal two-thirds of the mucosa was gently scraped and stored in RNAlater RNA stabilization reagent (QIAGEN GmbH, Germany) at −20℃.

Total RNA from tissue samples was extracted and purified using the RNeasy Lipid Tissue Mini Kit (cat. 70804; QIAGEN GmbH, Germany). One microgram of the total RNA was primed with 50 pmol of oligo (dT) 20 and transcripted with Superscript Ⅲ (Invitrogen, CA, USA) for first-strand cDNA synthesis, according to the manufacturer's protocol. qRT-PCR was performed; DNA polymerase and SYBR Green I (Finnzymes Oy, Espoo, Finland) were set in a reaction volume of 20 μL containing gene-specific primers (5 μM) and cDNA (corresponding to ~50 ng total RNA). The reaction was performed using the DNA engine Opticon 2 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The $2^{-\Delta\Delta CT}$ method of relative gene expression was employed and a standard deviation of ct value of <0.3 was accepted. Results are expressed as arbitrary units in comparison with the expression of GAPDH.

Primers for this Study

The sequence data of the genes were found in GenBank and the sequences of primers were designed with Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). GAPDH was used as a housekeeping gene. The sequence and information for primers used in this study are as follows: FATP-4 (GenBank accession number NM_011989): 5'-atcaacaccaaccttaggcg-3' and 5'-aacccttgtctgggtgactg-3', FABP1 (NM_017399): 5'-catccagaaagggaaggaca-3' and 5'-ttttcccc-agtcatggtctc-3', FABP2 (NM_007980): 5'-ttgctgtccgag-aggtttct-3' and 5'-gctttgacaaggctggagac-3', DGAT-1 (NM_010046): 5'-gtgcacaagtggtgcatcag-3' and 5'-cag-tgggatctgagccatc-3', DGAT-2 (NM_026384): 5'-agtg-gcaatgctatcatcatcgt-3' and 5'-aaggaataagtgggaacca-gatca-3', MGAT-2 (NM_177448): 5'-gaagaagcagcat-cagggac-3' and 5'-gtgtgggattaggggactt-3', ApoB (NM_009693): 5'-tgggattccatctgccatctcgag-3' and 5'-gtaga-gatccatcacaggacaatg-3', MTTP (NM_008642): 5'-cat-gtcagccatcctgtttg-3' and 5'-ctcgcgataccacagactga-3', and GAPDH (NM_008084): 5'-actccactcacggcaaa-ttc-3' and 5'-tctccatggtggtgaagaca-3'.

Statistical Analysis

The values are expressed as the means ± S.D. Statistical significance was assessed by Student's *t*-test for paired values and set at $p<0.05$.

Results

Fenofibrate Reduces Postprandial Hypertriglyceridemia, as well as ApoB-100 and ApoB-48 Mass in Wild-Type and CD36KO Mice in Fasting and Postprandial States

CD36KO mice showed significantly higher plasma TG levels than WT controls (638 ± 123 mg/dL vs. 168 ± 27 mg/dL, $p<0.05$) in the postprandial state (**Fig. 1**). Administration of fenofibrate decreased plasma TG concentrations in the fasting state in both WT (87 ± 32 vs. 21 ± 2 mg/dL, $p<0.05$) and CD36KO mice (82 ± 11 vs. 23 ± 4 mg/dL, $p<0.05$). Moreover, fenofibrate markedly reduced the postprandial plasma TG concentration in CD36KO mice (638 ± 123 vs. 45 ± 20 mg/dL, $p<0.05$), while the reduction of TG in WT mice was somewhat modest compared to that in CD36KO mice (168 ± 27 vs. 52 ± 14 mg/dL, $p<0.05$). This marked diminution of the TG level in the postprandial state in CD36KO mice after fenofibrate treatment implies that fenofibrate could act more efficiently in the postprandial state in the MetS environment. It is important to point out that fenofibrate administration did not affect mouse weight significantly during the 2-week treatment in both CD36KO

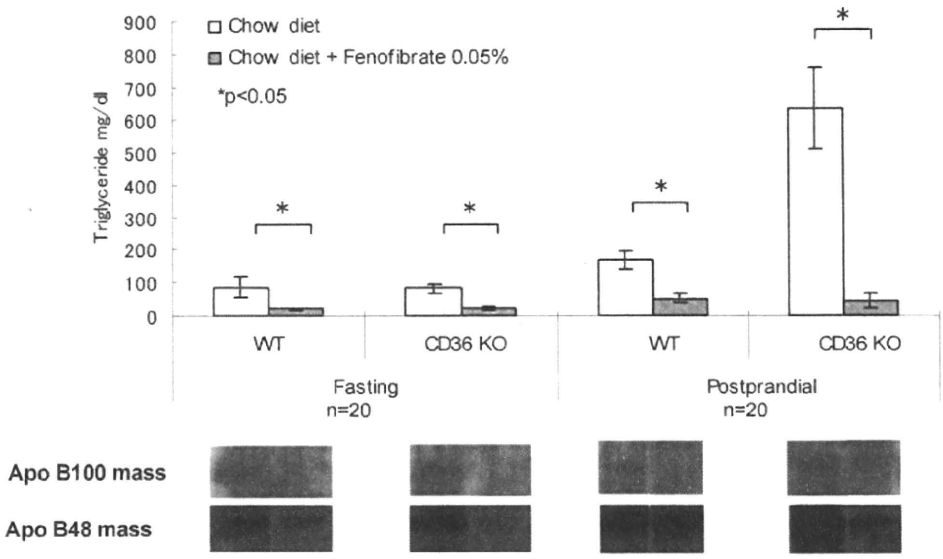

Fig. 1. Fenofibrate decreased plasma TG levels in CD36 knockout and WT mice in fasting and postprandial states.

(Upper panel) Addition of fenofibrate (gray) showed a significant decrease in TG levels in fasting and postprandial states in WT and CD36KO mice; the TG reductions for WT and CD36KO mice were 3.2 and 13.9 times, respectively. ($p < 0.05$)
(Lower panel) Representative Western blot images of apoB-100 and apoB-48 masses loaded the same amount of plasma in each subgroup.

and WT groups compared to their chow diet controls (data not shown).

To assess the effect of fenofibrate administration on apoB-48 mass in the plasma of WT and CD36KO mice in fasting and postprandial states, Western blotting was performed. The amount of both apolipoprotein B isoforms, apoB-100 and apoB-48, in plasma was markedly reduced after fenofibrate treatment in both states and strains (**Fig. 1**), implying that both apoB-100- and apoB-48-containing lipoproteins were reduced.

Fenofibrate Reduces Postprandial CM and VLDL-Sized Particles in Plasma of CD36KO Mice

The plasma lipoprotein profile was analyzed by automatic HPLC using a pool of 3 samples for each group. CD36KO mice showed a marked increase in postprandial TG levels of every lipoprotein fraction compared to their WT controls before fenofibrate administration. Among subfractions, a substantial difference between CD36KO and WT mice was demonstrated in TG levels of CM and VLDL-sized particles, which also include CM remnants, indicating that CD36KO mice showed impaired TG-rich lipoprotein metabolism in the postprandial state (**Fig. 2A**). Fenofibrate reduced postprandial TG levels in WT and CD36KO mice mainly in these subfractions (**Fig. 2B, 2C**). **Fig. 2D** shows the overall HPLC analysis of CD36KO mouse plasma in the postprandial state before and after fenofibrate treatment. These results raised the possibility that fenofibrate could modulate intestinal CM production. Thus, we further investigated the lipoproteins in the intestinal lymph and intestinal mRNA expression of genes in CD36KO mice in the postprandial state before and after fenofibrate treatment.

Fenofibrate Reduces Postprandial TG and ApoB-48 Mass in Intestinal Lymph of CD36KO Mice

Fenofibrate significantly reduced the postprandial TG concentration in the intestinal lymph of CD36KO mice in the postprandial state (18.6 ± 2.2 vs. 10.0 ± 1.6 g/dL, $p < 0.05$) accompanied by a decrease in ApoB-48 mass (**Fig. 3A**). The highest peak in TG levels corresponded to the CM fraction in both treated and non-treated mice, with a discrete elevation in the VLDL-sized fraction, which corresponds to CM remnants, since the obtained lymph lacked apoB-100 (**Fig. 3B**). Fenofibrate decreased both CM and CM remnant-sized curves, suggesting that fenofibrate might decrease the production of intestine-derived lipoproteins in the postprandial state in CD36KO mice (**Fig. 3B**).

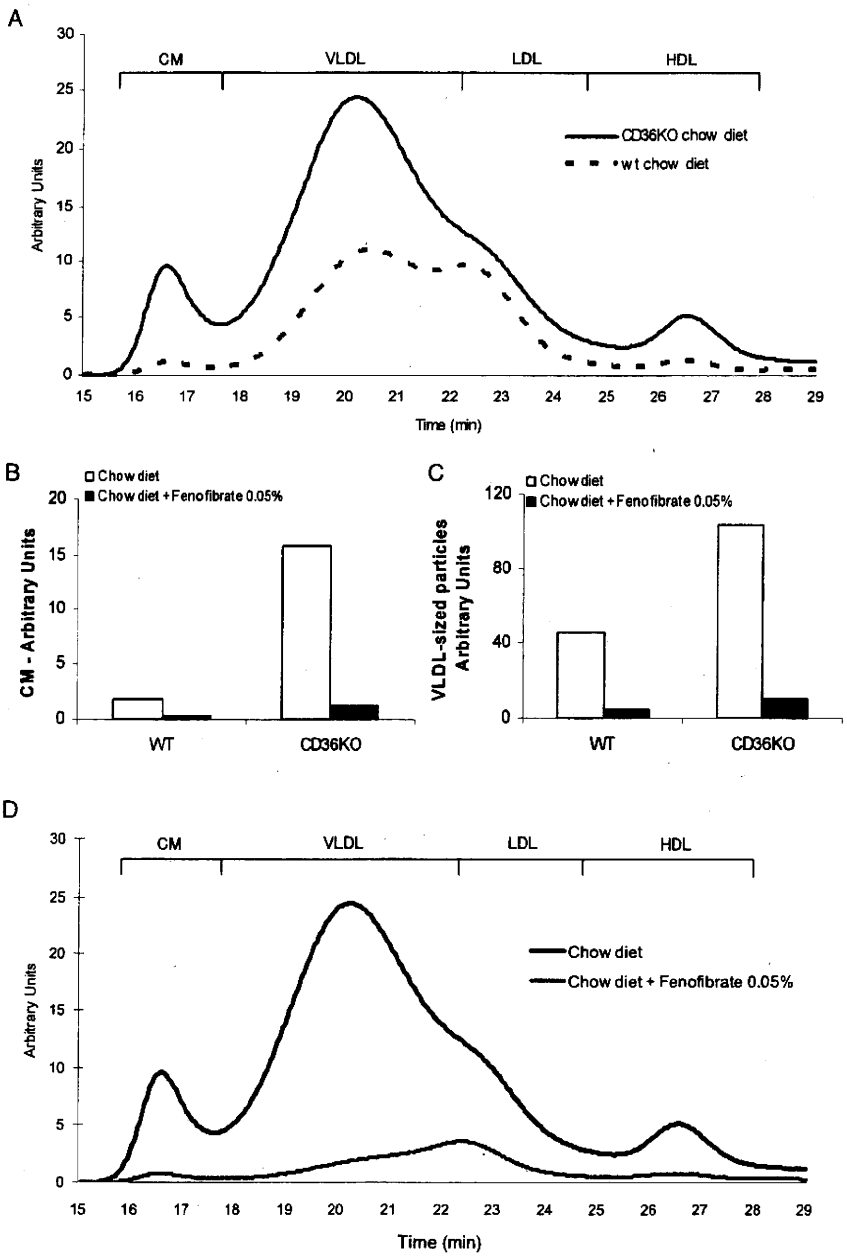

Fig. 2. Effects of fenofibrate on lipoprotein fractions in plasma of CD36KO mice in postprandial state.

(A) HPLC performed on plasma showed a higher 3-hour postprandial TG response of CD36KO (solid line) than WT mice (dashed line). Fenofibrate treatment (black) decreased postprandial plasma TG levels in CM (B) and VLDL-sized particles which also include CM remnants (C). (D) Plasma HPLC curves in postprandial state before (solid black line) and after (solid gray line) fenofibrate treatment in CD36KO mice

Fenofibrate is Involved in the Transcriptional Regulation of Lipid Metabolism-Related Genes in Intestine of CD36KO Mice in Postprandial State

To determine the possible mechanisms involved in the attenuation of postprandial hypertriglyceridemia by fenofibrate, qRT-PCR using isolated total intestinal mRNA was performed and the expression of genes associated with FA and TG transport as well as

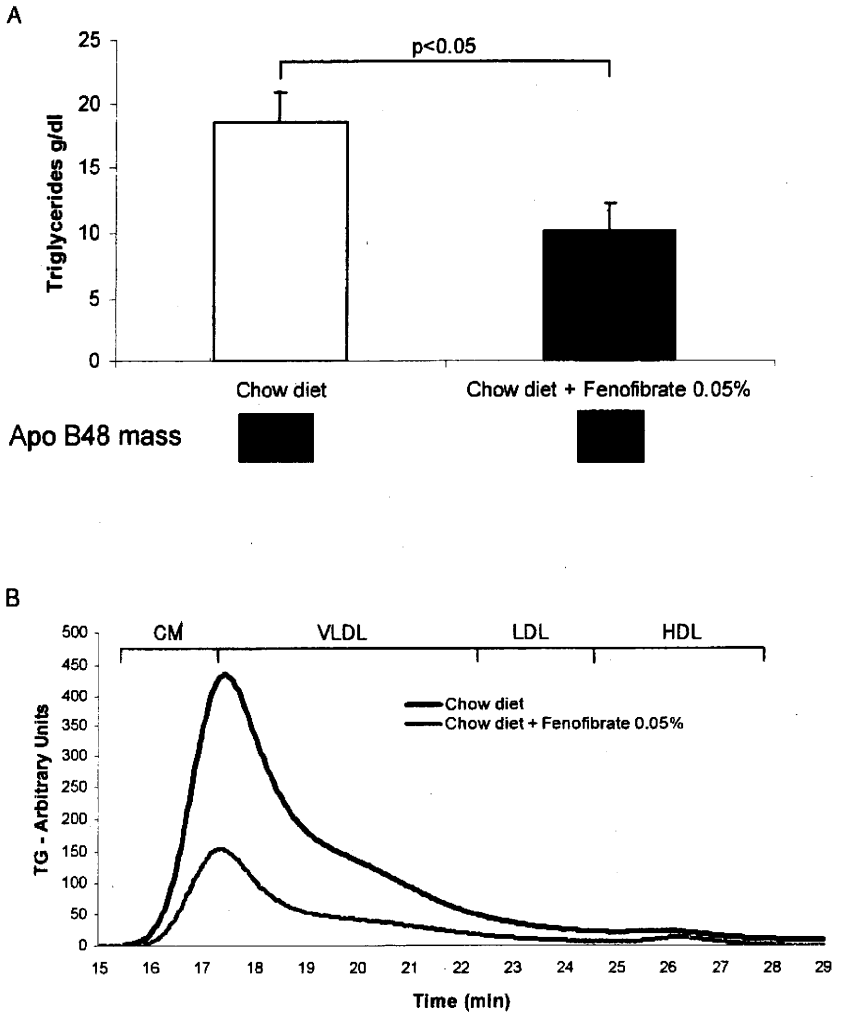

Fig. 3. Fenofibrate reduces postprandial TG and apoB-48 mass in intestinal lymph of CD36KO mice.

(A) Fenofibrate treatment (black bar) significantly reduced postprandial TG in intestinal lymph of CD36KO mice, and also notably decreased the apoB-48 mass 3 hours after the ingestion of a fat load. (B) HPLC curves of lymphatic lipoproteins in postprandial state before (solid black line) and after (solid gray line) fenofibrate treatment in CD36KO mice.

CM assembly in the intestine of CD36KO mice treated and non-treated with fenofibrate was examined. First we investigated the intestinal PPARalfa expression to confirm the efficacy of fenofibrate treatment in this experiment. Fenofibrate administrated for two weeks to CD36KO mice increased the intestinal mRNA expression of PPARalfa 2-fold.

The mRNA levels of fatty acid transport protein (FATP)-4, and fatty acid binding proteins (FABP)-1 and FABP-2, which are highly associated with the uptake and transport of long chain FAs, did not change significantly in the presence of fenofibrate. The mRNA expression of diacyl glycerol acyl transferase (DGAT)-1, DGAT-2, and monoacyl glicerol acyl transferase (MGAT)-2, which are involved in the intracellular formation of TG in intestinal epithelial cells, did not change significantly (**Fig. 4**).

ApoB mRNA was found to be decreased in mice fed with fenofibrate, while the genes that participate in apoB mRNA production, apobec-1 and apobec-1 complementation factor (ACF), were not affected significantly, which suggests the decrease of intestinal apoB mRNA as a determinant factor in the inhibitory action of fenofibrate on CM production (**Fig. 4**).

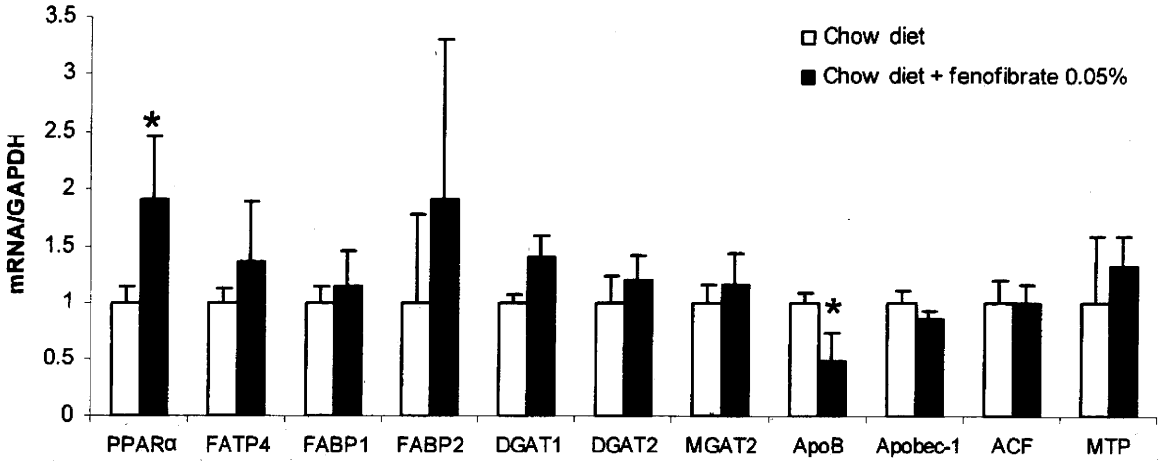

Fig. 4. Fenofibrate was involved in the transcriptional regulation of lipid metabolism-related genes in the intestine of CD36KO mice in postprandial state.

The mRNA expression of genes involved in intestinal TG manipulation and CM production were evaluated by qRT-PCR. Fenofibrate decreased apoB mRNA in CD36KO mice in postprandial state significantly ($p < 0.05$). No significant difference was observed in the expression of genes that regulate apoB mRNA production, as well as in those associated with fatty acid transport, TG formation, and CM assembly.

Interestingly, microsome triglyceride transfer protein (MTP) mRNA expression, considered to have an important role in CM assembly in epithelial cells, was not significantly altered by the presence of fenofibrate.

Discussion

The TG-lowering effect of fenofibrate has been widely reported to occur mainly via the activation of lipoprotein lipase (LPL) by increased hepatic LPL mRNA levels and by suppression of liver mRNA levels of apoCIII, which is a potent inhibitor of LPL. The former was supported by the finding of a peroxisome proliferator-response element (PPRE) in the human LPL gene[22]. Fenofibrate also down-regulates lipogenic genes in the liver, such as fatty acid synthase, acetyl CoA carboxylase, and DGAT-2, inducing hepatic FA uptake and reducing FA synthesis and VLDL production in hepatocytes, thereby directly affecting the catabolism of TG-rich lipoprotein[23, 24].

As described above, the mechanism of action of fenofibrate in the TG-lowering effect was largely centered on the liver and could explain in part the marked reduction of VLDL-sized CM remnants observed in the plasma of treated CD36KO mice (**Fig. 2D**). However, little is known about the effect of fenofibrate on TG metabolism in the intestine. We did not determine LPL activity in our study, already mentioned as a crucial factor in the TG-lowering effect of fenofibrate, since we focused on the mechanisms concerning the intestinal production of ApoB-containing lipoproteins. This study added a novel mechanism of the TG-lowering effect of fibrates, that is, the production of intestine-derived lipoproteins, CM and CM remnant-sized particles, was inhibited by fenofibrate (**Fig. 3B**).

It is known that CD36KO mice present an increased hypertriglyceridemic response to both oral fat loading and chronic exposure to a high fat diet compared to WT mice[14]. Our laboratory previously found an increased TG concentration and apoB-48 mass in the intestinal lymph of CD36KO mice in fasting and postprandial states, without any alteration in lipoprotein lipase (LPL) or hepatic lipase activity between CD36KO and WT mice, highly suggesting that the postprandial hypertriglyceridemia observed in this animal model is due to increased CM production from the intestine[15]. In the present study, we demonstrated that the PPAR-α agonist fenofibrate was able to decrease postprandial TG levels in plasma and intestinal lymph of CD36KO mice.

Our results also showed a statistically significant reduction in the postprandial apoB mRNA expression of CD36KO mice treated with fenofibrate, which might suggest this as the mechanism responsible for reduced CM production. However, the regulation of apoB has been largely reported to be posttranscriptional, although it is also true that most of these studies were not performed in intestinal cells but in hepa-

tocytes[25]. Fu *et al.*[26] reported the PPAR-α agonist ciprofibrate as an inhibitor of the expression of ACF, one of the responsible factors of apoB mRNA production; however, this inhibition was found only in the liver, not in the intestine of LDL-receptor knockout mice. This supports the idea that the factors involved in the regulation of apoB lipoproteins, including PPAR-α agonists, might differ between these two tissues, which leads to the need for more studies to understand the regulation of apoB in the small intestine.

MTP catalyzes the transfer of TG and cholesteryl esters to apoB and therefore has a main role in the assembly of apoB-containing lipoproteins. It has been reported that PPAR-α agonists increase MTP expression and apoB secretion in rodent liver but not in the intestine in spite of decreased plasma TG levels[27]. We found that MTP expression was not affected by fenofibrate in the intestine of CD36KO mice in the postprandial state, which also contributes to the idea that regulation of the production of apoB-containing lipoproteins in the intestine might be different from the liver.

Our results show fenofibrate to be an effective treatment for postprandial hyperlipidemia in CD36KO mice, and the reduction in the intestinal production of ApoB-containing lipoproteins as a new mechanism of action for this drug. Thus, since human CD36 deficiency is a genetic background of metabolic syndrome, as stated previously, we suggest that fenofibrate might play a similar role not only in CD36-deficient patients, but in MetS; this hypothesis, however, needs to be tested in further studies.

Conclusion

Fenofibrate reduces postprandial hypertriglyceridemia in CD36 knockout mice; this reduction is associated with the inhibition of intestinal apoB-48 production and the subsequent reduction of intestinal apoB-containing lipoproteins. This suggests a protective effect of fenofibrate against atherosclerosis in CD36KO mice as a monogenic model of metabolic syndrome.

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Original Article

Molecular Mechanisms of Ezetimibe-Induced Attenuation of Postprandial Hypertriglyceridemia

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Aim: Postprandial hypertriglyceridemia (PHTG) has been shown repeatedly to be associated with metabolic syndrome and atherosclerotic cardiovascular diseases. We have recently reported that ezetimibe inhibits PHTG in patients with type IIb hyperlipidemia. Ezetimibe was also reported to attenuate PHTG in combination with low-dose statins in patients with obesity or metabolic syndrome. We reported CD36-deficient (CD36KO) mice as a new model for PHTG, in which the synthesis of chylomicron (CM) in the small intestines is enhanced. In the current study, we investigated the effect of ezetimibe on PHTG in this mouse model of metabolic syndrome.

Methods: Wild-type (WT) mice fed a western diet, and CD36KO mice fed a normal chow diet, respectively, were treated for 3 weeks with and without ezetimibe, followed by an evaluation of triglyceride (TG) concentrations by enzymatic method and by high performance liquid chromatography (HPLC) as well as those of and apolipoprotein (Apo) B-48 in plasma and intestinal lymph after oral fat loading with olive oil. Intestinal mucosa was also harvested to evaluate the transcriptional regulation of the genes involved in the intestinal production of ApoB-containing lipoproteins.

Results: Ezetimibe dramatically reduced PHTG in both WT and CD36KO mice. HPLC analysis of plasma showed that the decrease in TG content in CM and CM remnants-sized particles contributed to this suppression, suggesting that CM production in the small intestines might be reduced after ezetimibe treatment. Intestinal lymph was collected after oral fat loading in ezetimibe-treated and non-treated mice. Both TG content and ApoB-48 mass were decreased in ezetimibe-treated mice. The quantitative RT-PCR of intestinal mucosa showed down-regulation of the mRNA expression of FATP4 and ApoB in both groups along with FABP2, DGAT1, DGAT2 and SCD1 in WT mice at postprandial state after ezetimibe treatment.

Conclusion: Ezetimibe alone reduces PHTG by blocking both the absorption of cholesterol and the intracellular trafficking and metabolism of long-chain fatty acids in enterocytes, resulting in the reduction of the formation of ApoB-48 which is necessary for the ApoB48-containing lipoprotein production in the small intestines.

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Key words; Postprandial hypertriglyceridemia, Ezetimibe, CD36 deficiency, Long-chain fatty acids, Apolipoprotein B-48

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Introduction

Metabolic syndrome (MetS) is defined as a cluster of interrelated factors commonly associated with atherosclerotic cardiovascular diseases: central obesity, modestly high blood pressure, impaired glucose