■ 治療

高HDLコレステロール血症患者を診た場合には成因を検索するとともに，狭心症の有無を聴取し，負荷心電図，頸動脈超音波検査などを施行する．薬物療法に関して一定の見解はないが，動脈硬化を合併する場合にはほかの危険因子の軽減に努める．

■ 経過・予後

高HDLコレステロール血症の原因となる疾患や動脈硬化性疾患の合併の有無によって経過や予後が規定される．

〔山下静也〕

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先天性脂質代謝異常
inborn errors of lipid metabolism

■ 概念

遺伝子変異により脂質代謝異常を起こす疾患には，血清脂質の量に大きな変化を生じる脂質異常症（高脂血症）や低脂血症があるが，そのほかに脂質が組織や細胞内に蓄積して多彩な全身症状を引き起こすまれな疾患群がある．

大部分は，細胞のオルガネラの一つであるリソソームに基質が蓄積して機能障害を起こすので，まとめてリソソーム病（lysosomal disease）あるいはリソソーム蓄積症（lysosomal storage disease）と呼ぶ．

これまでに脂質と糖質の蓄積症が知られている．

蓄積する脂質は分子内に糖質を有する糖脂質，特にスフィンゴ脂質が多く，まとめてスフィンゴリピドーシス（sphingolipidosis）と呼ばれる．

ほかに中性脂質の蓄積症（Wolman病など）もみられる．

■ 分類

表47に示すとおり，蓄積する脂質によりスフィンゴリピドーシス，中性脂肪蓄積症と脂肪酸蓄積症に大別される．スフィンゴリピドーシスはさらに，①ガングリオシドーシス（gangliosidosis），②ミエリン脂質代謝異常，③その他，に分類できる．

特にガングリオシドは中枢神経系に多く存在し，分子内にシアル酸をもつため，脂質であるにもかかわらず親水性が高い．細胞内や細胞表面で種々の生理活性をもつ．その蓄積症（ガングリオシドーシス）を含む多くのスフィンゴリピドーシスは，小児期に強い進行性の脳症状を起こす．ミエリン脂質の代謝異常は，脳白質に主病変があり，白質ジストロフィ（leukodystrophy）と呼ぶ．

ミトコンドリアやペルオキシソームの酵素欠損により，コレスタノールや脂肪酸が特定の組織に蓄積する病気も知られている．

■ 疫学

これらの遺伝性脂質蓄積症はまれな疾患で，正確な発生頻度は不明であるが，出生10万から100万に1例程度と予想される．遺伝形式は，大部分が常染色体劣性遺伝であるが，Fabry病と副腎白質ジストロフィのみ伴性（X染色体関連）劣性遺伝で，原則として男性のみに発症する．

■ 病因

リソソームに存在する加水分解酵素の遺伝性欠損による．リソソームは成熟赤血球以外，すべての体細胞に存在するので，すべての細胞に基質が蓄積する可能性がある．しかし実際には，その脂質の細胞内代謝の活発な組織に病変が発生する．

表47の欠損酵素をコードする遺伝子はほとんどすべてクローニングが行われ，塩基構造の異常部位も明らかにされている．変異には，ある特定の人種に共通のものもあるが，家系ごとにまったく異なる多様な変異を示す病気もある．

■ 病理

病変が強く発現する組織は疾患ごとに異なるが，一般に細胞は膨満し，蓄積する脂質により特有の形態を示す．

Gaucher病，Niemann-Pick病には骨髄に大型の蓄積細胞（Gaucher細胞，Niemann-Pick細胞）が，

Original Article

Significance of Measuring Serum Concentrations of Remnant Lipoproteins and Apolipoprotein B-48 in Fasting Period

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Aim: To characterize lipid profiles conveniently in the fasting period to detect postprandial hyperlipidemic subjects, we measured the concentrations of lipids, including remnant lipoproteins and apoB-48, before and after loading the test meal in 24 normolipidemic subjects.
Methods: We examined remnant-like particle-cholesterol and -triglyceride (RLP-C, RLP-TG) by the immune adsorption method, RemL-C by the newly developed homogeneous method, and apoB-48 by chemiluminescence enzyme immunoassay.
Results: After loading, TG, RemL-C, RLP-C, RLP-TG, and apoB-48 concentrations were elevated. Twenty subjects had only a slight elevation of TG (low TG group) after loading, while 4 subjects showed apparent increase of TG (more than 150 mg/dL, high TG group). In the fasting period, the high TG group had significantly higher serum concentrations of TG and RemL-C than the low TG group. Although not significant, RLP-C, RLP-TG and apoB-48 concentrations in the high TG group were also higher than in the low TG group. After loading, serum concentrations of TG, RemL-C, RLP-C, RLP-TG, and apoB-48 increased significantly more in the high TG group than in the low TG group.
Conclusion: In conclusion, TG, RemL-C, RLP-C, RLP-TG, and apoB-48 concentrations in the fasting period may be suitable for detecting postprandial hyperlipidemic subjects.

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Key words; Postprandial hyperlipidemia, RLP-C, RemL-C, Small, dense LDL-C

Introduction

Since Zilversmit's proposal of the significance of postprandial hyperlipidemia, many studies have investigated the role of remnant lipoproteins in the pathogenesis of atherosclerosis, and have identified a delay in their removal from blood as an independent risk factor[1-6]. In fact, high concentrations of remnant-like particle (RLP)-cholesterol (RLP-C) predict coronary events in patients with CAD, independent of traditional coronary risk factors[7-9].

In normal humans, the postprandial hyperlipidemic period is about 4-6 hours, but in individuals with certain dyslipidemia, this period may be increased beyond 6 hours[3,4]. Of note, impaired removal of chylomicron remnants in the liver potentially induces longer retention times for these lipoproteins in blood circulation. Moreover, it is important to recognize that dietary lipid is transferred to various parts of the body via plasma lipoproteins after food ingestion.

In the postprandial period, we can observe the elevation of triglyceride (TG)-rich lipoproteins, which include chylomicrons, very-low-density lipoproteins

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(VLDL), and their remnants, in the blood circulation. Nascent chylomicrons, synthesized by enterocytes, have a high TG-to-cholesterol mass ratio, and consist primarily of apolipoprotein (apo) B-48 and apoA-I [10, 11]. After acquiring apoC-II and apoE, chylomicrons bind to lipoprotein lipase (LPL), which induces lipolysis of TG in chylomicrons. TG depletion results in a size reduction, and is referred to as chylomicron remnants, or "remnants". Finally, chylomicron remnants are cholesteryl ester-rich and retain apoB-48 and apoE[12, 13]. VLDL produced by hepatocytes are also hydrolyzed by LPL like chylomicrons, and become VLDL remnants containing apoB-100 and apoE.

Although it has been difficult clinically to distinguish exogenous lipids (chylomicrons and their remnants) from endogenous lipids (VLDL and their remnants), it recently became possible to conveniently measure the serum B-48 concentration [14]. In the present study, to characterize the lipid profiles conveniently in the fasting period to detect postprandial hyperlipidemic subjects, we measured the concentrations of lipids, including remnant lipoproteins and apoB-48, before and after loading the test meal in normolipidemic subjects.

Subjects and Methods

Subjects and Physical Examination

We recruited healthy subjects [n=24; male/female, 11/13; mean ± standard deviation (SD), 21.5 ± 1.2 years old] who had never been treated or taken any drugs at least 3 months before the study. All subjects gave their informed consent to participate in the study. The study protocol was carried out according to the Declaration of Helsinki.

Blood pressure was measured twice by the same observer using a standard mercury sphygmomanometer after the subject had rested in a supine position for 30 min. Waist circumference of subjects was measured [15]. Body mass index (BMI) was calculated by dividing body weight by the square of the height (kg/m^2).

Study Protocol

We used a test meal containing carbohydrate, fat, and protein, which was developed for the assessment of both postprandial hyperglycemia and hyperlipidemia by the Japanese Diabetes Society (Test meal A). This test meal consists of cream of chicken soup, biscuit, and custard pudding. The total calories is 450 kcal, including carbohydrate 57.6 g (51.4% in energy balance), protein 17.2 g (15.3%), fat 16.6 g (33.3%), which is a slightly higher percentage of fat than in the usual Japanese breakfast (20-25%). Blood samples were obtained at 9-10 AM after a 12-hour fast and 1, 2, 4, 6, and 8 hours after ingestion of the test meal.

Blood Sampling and Analysis

Serum concentrations of TG, total cholesterol, high-density lipoprotein (HDL)-cholesterol (HDL-C) and low-density lipoprotein (LDL)-cholesterol (LDL-C) were determined by enzymatic methods (Kyowa Medex, Tokyo, Japan): plasma oxidized LDL concentration by enzyme-linked immunosorbent assay (Kyowa Medex); serum concentrations of apoA-I, apoA-II, apoB, apoC-II, apoC-III, and apoE by turbidimetric immunoassay methods (Nittobo, Tokyo, Japan); serum apoB-48 concentration by chemiluminescence enzyme immunoassay (Fujirebio, Tokyo, Japan) [14]; serum small, dense-LDL-cholesterol (sd-LDL-C) concentration by the precipitation method (Denka Seiken, Tokyo, Japan) [16]; serum concentrations of RLP-C and RLP-TG by the immune adsorption method (JIMRO II, Otsuka Pharmaceutical, Tokyo, Japan) [17]; serum remnant lipoprotein cholesterol (RemL-C) concentration by homogenenous assay (MetaboLead RemL-C, Kyowa Medex) [18]; serum high sensitivity C-reactive protein (hs-CRP) concentration by nephelometry method (Dade Behring, Deerfield, IL); plasma glucose concentration and glycosylated hemoglobin A1C (HbA1C) by HPLC; and serum insulin concentrations by enzyme immunoassay, respectively.

Agarose Gel Electrophoresis Analysis

Samples were subjected to lipoprotein analysis using agarose gel electrophoresis (Rapid Electrophoresis; Helena Laboratories, Beaumont, Texas), with 15 min of electrophoresis at 400 volts and 20℃. After staining with cholesterol and TG reagent, elution profiles were analyzed by an automatic densitometer, Chol/Trig Combo™ (Helena Kenkyusho, Saitama, Japan) [19]. Contents of cholesterol and TG in each fraction were calculated with total lipids and the area under the curve according to the report by Kido *et al.* [20]. Moreover, the ratios of cholesterol to TG in HDL and LDL fractions were calculated and compared with those in healthy volunteers reported previously (mean ± SD; HDL, 5.8 ± 2.0; LDL, 4.9 ± 1.3) [21].

Statistical Analysis

Values are expressed as the mean ± SD. Statistical significance of data was evaluated using either the Mann-Whitney U-test or Welch's t-test. Correlations between apoB-48 and other parameters were calculated using the formula for Pearson's correlation coefficient. Responses to the test meal were compared by analysis of variance (ANOVA) for repeated measures.

Table 1. Glucose and lipid parameters before and after loading the test meal

	0	1h	2h	4h	6h	8h
Plasma glucose (mg/dL)	89.4±4.7	104.5±19.2**	89.2±11.5	85.3±4.8**	85.2±4.5**	85.1±5.0**
Insulin (μU/mL)	6.1±2.9	53.7±27.3**	24.6±16.4**	5.1±2.2	4.1±1.3*	3.7±1.4*
TG (mg/dL)	65.6±25.5	86.9±38.2*	95.7±47.4*	77.1±32.6	60.7±19.2	52.2±16.8*
TC (mg/dL)	182.6±32.2	181.5±33.8	179.8±32.4	181.4±30.8	184.2±31.7	189.5±33.3
LDL-C (mg/dL)	100.0±25.6	97.8±25.7	96.8±25.4	97.9±24.4	101.1±25.2	104.3±26.2
HDL-C (mg/dL)	70.0±14.3	67.8±14.2	67.2±13.1	68.0±14.1	70.1±14.9	72.5±15.2
RemL-C (mg/dL)	3.5±1.6	3.9±1.9	4.0±2.1	3.7±2.1	3.1±1.3	2.9±1.1
RLP-C (mg/dL)	3.1±1.2	4.4±2.0*	4.7±2.6*	3.8±1.7	3.0±1.3	2.8±0.9
RLP-TG (mg/dL)	15.8±2.6	24.8±14.6*	30.4±23.6*	20.1±8.7	15.2±0.7*	15.1±0.2*
Sd-LDL-C (mg/dL)	21.4±8.9	17.3±5.8	17.1±6.9	17.0±5.7	17.5±5.9	17.8±5.8
Oxidized LDL (U/mL)	6.7±4.9	6.1±4.4	6.6±5.5	6.5±4.8	6.8±4.7	7.1±4.9
ApoA-I (mg/dL)	164.0±27.0	161.8±28.0	161.3±25.0	162.5±26.7	165.5±26.7	168.2±28.1
ApoA-II (mg/dL)	38.9±7.1	38.5±7.4	38.0±6.9	38.5±7.0	38.7±7.1	39.3±7.1
ApoB (mg/dL)	67.5±14.2	66.3±14.8	66.2±14.0	66.8±13.6	68.5±13.9	70.5±14.2
ApoB-48 (μg/mL)	3.2±2.1	6.2±3.1**	6.1±3.4**	5.2±2.9**	3.6±2.1	3.0±1.7
ApoC-II (mg/dL)	3.1±1.1	3.2±1.2	3.2±1.2	3.2±1.1	3.2±1.1	3.2±1.1
ApoC-III (mg/dL)	9.4±2.5	9.9±2.6	9.5±2.6	9.3±2.4	9.1±2.3	9.3±2.4
ApoE (mg/dL)	4.6±1.0	4.5±1.0	4.4±1.0	4.4±1.0	4.3±0.9	4.4±0.9

Values are expressed as the mean ± SD, *$p<0.05$, **$p<0.01$ (vs. 0 time) by Mann-Whitney U-test. TG: triglyceride, TC: total cholesterol, LDL-C: low-density lipoprotein-cholesterol, HDL-C: high density lipoprotein-cholesterol, RemL-C: remnant lipoprotein cholesterol measured with "Metabo-Lead RemL-C", RLP-C: remnant-like particle-cholesterol measured with "JIMRO II", RLP-TG: remnant-like particle-triglyceride, Sd-LDL-C: small, dense-LDL-cholesterol, Apo: apolipoprotein.

Data under the threshold of RLP-C (<2.0 mg/dL) or RLP-TG (<15 mg/dL) were treated as 2.0 mg/dL or 15 mg/dL, respectively. Statistical analysis was performed using Stat Flex ver.5.0 software (Artec, Osaka, Japan). Two-tailed values of $p<0.05$ were considered significant.

Results

Characteristics of Subjects

Subject characteristics are as follows (mean ± SD): BMI, 20.7±1.7 kg/m^2; waist circumferences, 72.3±4.2 cm in men and 65.7±4.5 cm in women; HbA$_{1c}$, 4.9±0.2%; hsCRP, 0.04±0.02 mg/dL.

Fasting and Postprandial Concentrations of Lipids in Total Subjects

Table 1 shows the changes of lipid concentrations before and after loading the test meal in all subjects. In the fasting period (time 0), concentrations of all parameters were within normal limits or low ranges; however, there were significant correlations between apoB-48 concentration and TG, RemL-C, RLP-C, RLP-TG, apoC-II, or apoC-III concentration (**Table 2**), indicating that intestine-derived lipoproteins were present in the circulation and had characteristics of remnants even in the fasting period in normolipidemic subjects.

After loading the test meal, TG, RLP-C, RLP-TG, and apoB-48 concentrations elevated significantly compared with before loading (**Table 1**). TG, RLP-C, and RLP-TG concentrations peaked at 2 hours, and were restored to the baseline within 4 hours. ApoB-48 concentrations peaked at 1 hour, and returned to basal levels at 6 hours. RemL-C concentrations also peaked at 2 hours and were restored within 6 hours, but this elevation had no significance. On the other hand, the concentrations of TC, HDL-C, LDL-C, sd-LDL-C, oxidized LDL, apoA-I, apoA-II, apoB, apoC-II, apoC-III, and apoE were not elevated. Sd-LDL-C concentrations decreased below the basal levels during the study without statistical significance.

Comparison of Fasting Lipid Concentrations between High and Low TG Groups

In the results of the loading test, we noticed that some subjects showed apparent increases of TG at 2 hours as peak values, and others showed only a slight elevation. We therefore established two groups by TG values at 2 hours, and designated subjects with <150 mg/dL of TG ($n=20$) as the low TG group and subjects with >150 mg/dL of TG ($n=4$) as the high TG

Table 2. Correlation between apoB-48 concentration and other parameters in fasting period

	r	*p*
Plasma glucose (mg/dL)	0.236	0.2661
Insulin (μU/mL)	0.042	0.8448
TG (mg/dL)	0.791	<0.0001
TC (mg/dL)	0.287	0.1743
LDL-C (mg/dL)	0.301	0.1526
HDL-C (mg/dL)	−0.081	0.7065
RemL-C (mg/dL)	0.811	<0.0001
RLP-C (mg/dL)	0.768	<0.0001
RLP-TG (mg/dL)	0.745	<0.0001
Sd-LDL-C (mg/dL)	0.367	0.0776
Oxidized LDL (mg/dL)	−0.150	0.4832
ApoA-I (mg/dL)	0.016	0.9395
ApoA-II (mg/dL)	0.290	0.1693
ApoB (mg/dL)	0.282	0.1820
ApoC-II (mg/dL)	0.689	0.0002
ApoC-III (mg/dL)	0.534	0.0071
ApoE (mg/dL)	0.189	0.3764
hs-CRP (mg/L)	0.253	0.2321

Correlations between apoB-48 and other parameters were calculated using the formula for Pearson's correlation coefficient. TG: triglyceride, TC: total cholesterol, LDL-C: low-density lipoprotein-cholesterol, HDL-C: high density lipoprotein-cholesterol, RemL-C: remnant lipoprotein cholesterol measured with "MetaboLead RemL-C", RLP-C: remnant-like particle-cholesterol measured with "JIMRO II", RLP-TG: remnant-like particle-triglyceride, Sd-LDL-C: small, dense-LDL-cholesterol, Apo: apolipoprotein, hs-CRP: high sensitivity-C-reactive protein.

Table 3. Comparison of clinical characteristics and fasting concentrations of glucose and lipid parameters between two groups

	High TG group (*n*=4)	Low TG group (*n*=20)	*P*
Age (years old)	22.0 ± 2.5	21.4 ± 0.9	0.6695
BMI (kg/m²)	22.0 ± 2.2	20.4 ± 1.5	0.2975
Abdominal circumference (cm)	73.5 ± 5.7	67.8 ± 5.2	0.1334
Hs-CRP (mg/dL)	0.06 ± 0.02	0.04 ± 0.02	0.1655
HbA1c (%)	5.0 ± 0.2	4.8 ± 0.2	0.3018
Plasma glucose (mg/dL)	83.0 ± 4.8	91.0 ± 3.6	0.0719
Insulin (μU/mL)	6.3 ± 2.6	6.1 ± 3.0	0.9060
TG (mg/dL)	109.5 ± 15.8	56.9 ± 16.1	0.0044
TC (mg/dL)	196.5 ± 14.6	179.9 ± 34.3	0.1460
LDL-C (mg/dL)	110.3 ± 16.6	98.0 ± 26.8	0.2982
HDL-C (mg/dL)	67.5 ± 26.7	70.5 ± 11.5	0.8477
RemL-C (mg/dL)	5.9 ± 1.3	3.0 ± 1.2	0.0148
RLP-C (mg/dL)	4.9 ± 1.4	2.8 ± 0.8	0.0694
RLP-TG (mg/dL)	19.5 ± 5.4	15.6 ± 2.7	0.2744
Sd-LDL-C (mg/dL)	30.7 ± 13.0	17.9 ± 5.6	0.1602
Oxidized LDL (U/mL)	4.9 ± 1.7	7.0 ± 5.2	0.1630
ApoA-I (mg/dL)	163.8 ± 49.1	164.0 ± 22.5	0.9930
ApoA-II (mg/dL)	44.0 ± 10.4	37.9 ± 6.0	0.3609
ApoB (mg/dL)	75.8 ± 10.2	65.8 ± 14.5	0.1865
ApoB-48 (μg/mL)	6.1 ± 2.6	2.6 ± 1.5	0.0957
ApoC-II (mg/dL)	3.6 ± 1.1	2.9 ± 1.1	0.3734
ApoC-III (mg/dL)	11.3 ± 3.7	9.1 ± 2.1	0.3531
ApoE (mg/dL)	5.1 ± 1.1	4.5 ± 0.9	0.4477

Values are expressed as the mean ± SD, Welch's *t* test. High group: the peak triglyceride (TG) concentration ≥150 mg/dL, low group: peak TG concentration <150 mg/dL, BMI: body mass index, HbA1c: glycosylated hemoglobin A1c, TC: total cholesterol, RemL-C: remnant lipoprotein cholesterol measured with "MetaboLead RemL-C", RLP-C: remnant-like particle-cholesterol measured with "JIMRO II", RLP-TG: remnant-like particle-triglyceride, Sd-LDL-C: small, dense-LDL-cholesterol, Apo: apolipoprotein.

group. The peak concentrations of TG in the low TG group were 78.4 ± 27.6 mg/dL, whereas those in the high TG group were 182.5 ± 33.7 mg/dL.

First, we compared the profiles and lipid parameters in the fasting period between the two groups (**Table 3**). The high TG group had significantly higher serum concentrations of TG and RemL-C than the low TG group. Although not significant, RLP-C, RLP-TG and apoB-48 concentrations in the high TG group were also higher than in the low TG group. There were no significant differences in the profiles, including age, BMI, and abdominal circumference between the two groups.

Comparison of Postprandial Concentrations between High and Low TG Groups

Fig. 1 demonstrates the sequential changes of parameters in lipids before and after loading the test meal in each group. Serum concentrations of TG, RemL-C, RLP-C, RLP-TG, and apoB-48 significantly increased more in the high TG group than in the low TG group, especially from 1 to 4 hours. In contrast, although sd-LDL-C concentrations were significantly higher in the high TG group, they gradually decreased after loading. There were no significant differences in serum concentrations of TC, LDL-C, HDL-C, and other apolipoproteins. Concentrations of glucose, insulin, and oxidized LDL also did not alter (data not shown).

Analysis of Lipid by Electrophoretogram

Fig. 2 demonstrates representative cases of densitometric scanning patterns of electrophoretogram and lipid data before and after loading the test meal in the high and low TG groups. Sample A belongs to the high TG group. Fractions of chylomicrons and VLDL

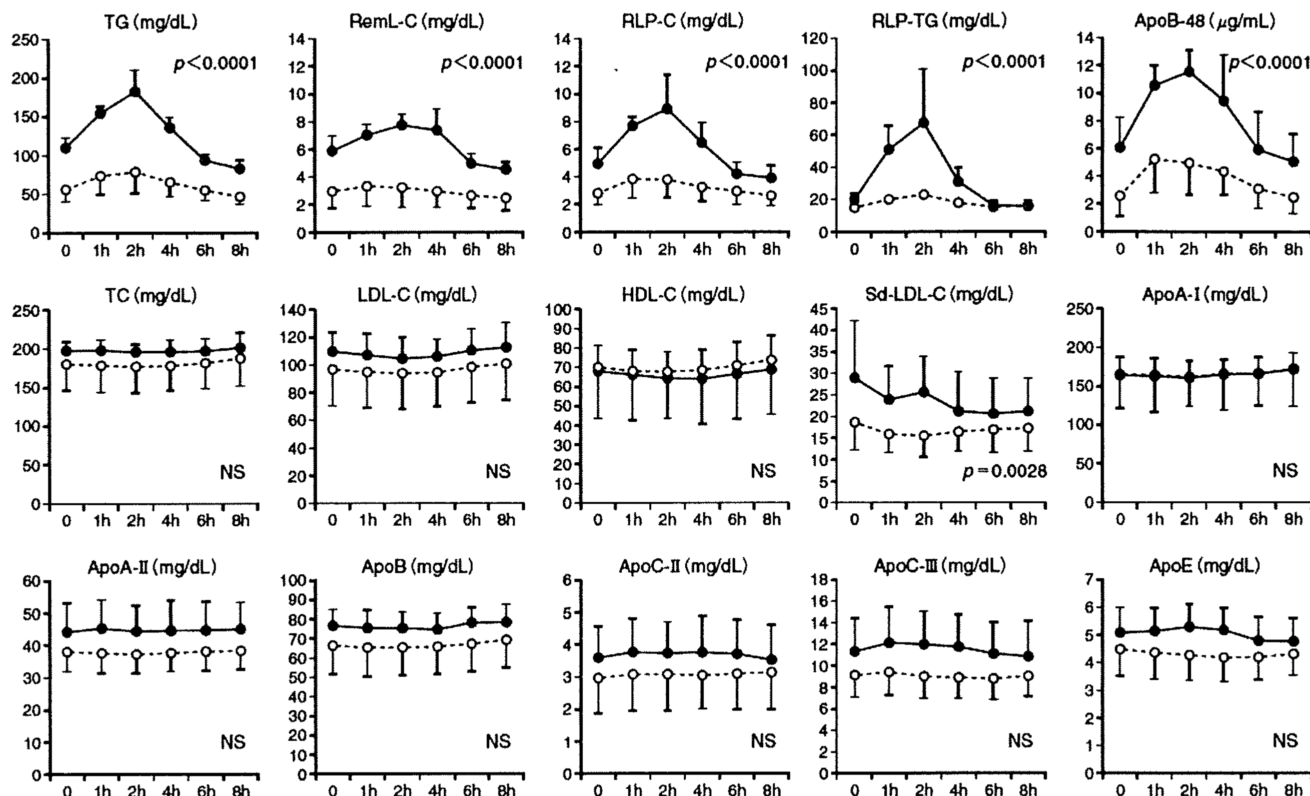


Fig. 1.

Changes in the levels of glucose and lipid parameters in subjects with <150 mg/dL (low TG group) and >150 mg/dL triglyceride concentration (high TG group) before and after loading the test meal. Responses to the test meal were compared by analysis of variance (ANOVA) for repeated measures. Open circle and broken line: low TG group, closed circle and black line: high TG group, TG: triglyceride, RemL-C: remnant lipoprotein cholesterol measured with "MetaboLead RemL-C", RLP-C: remnant-like particle-cholesterol measured with "JIMRO II", RLP-TG: remnant-like particle-triglyceride, TC: total cholesterol, HDL-C: high-density lipoprotein-cholesterol, LDL-C: low-density lipoprotein-cholesterol, sd-LDL-C: small, dense-LDL-cholesterol.

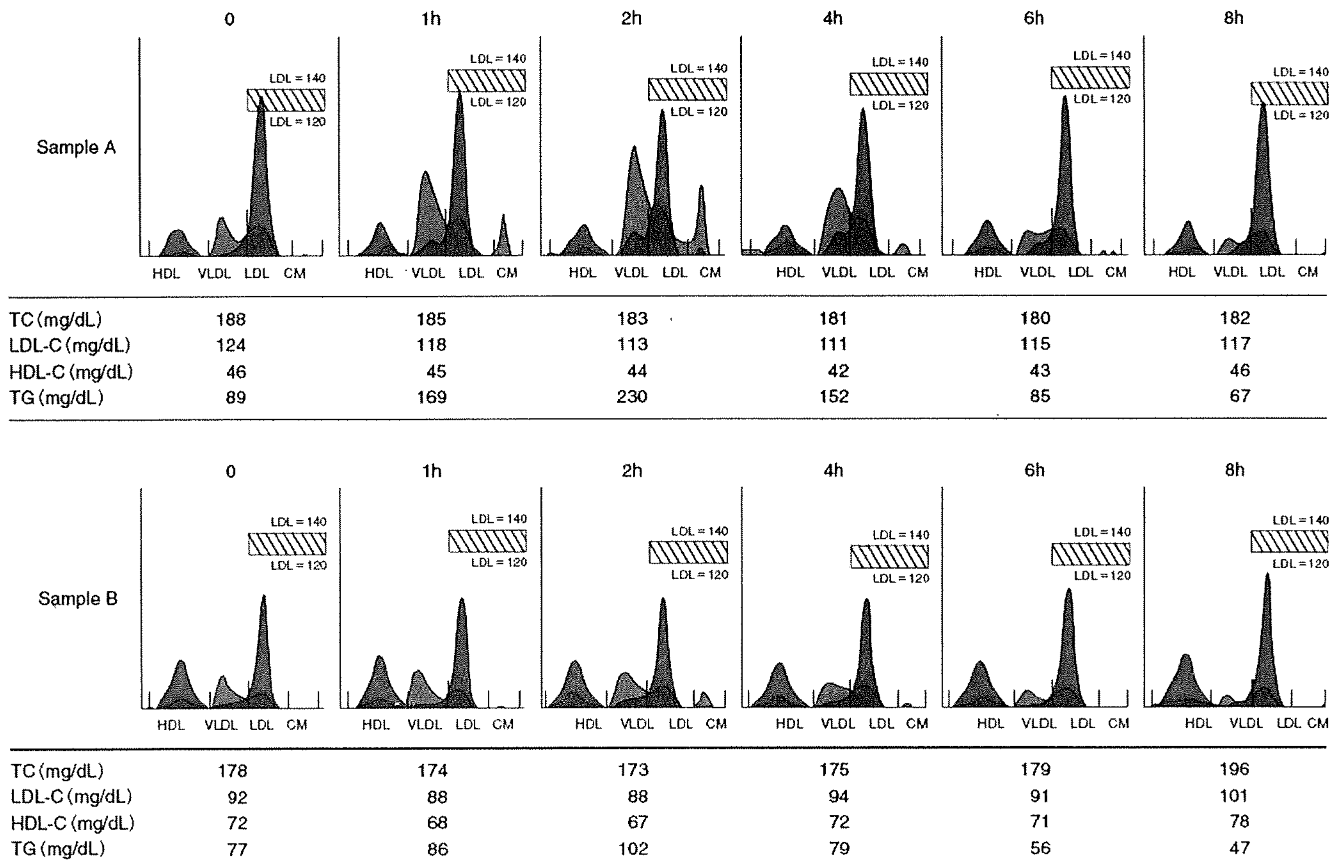
increased 1 hour after loading, peaked at 2 hours, and gradually decreased. Sample B belongs to the low TG group. We observed a slight increase of the chylomicron fraction 2 hours after loading. Thus, the peak values of TG and lipoprotein profiles in the postprandial period were different between the two cases, although fasting TG concentrations in both cases were around 80 mg/dL.

Fig. 3 shows the changes in TG concentrations of chylomicrons and VLDL, and the ratios of cholesterol to TG in HDL and LDL before and after loading the test meal. In the left panel of sample A, the higher the TG concentrations of chylomicrons and VLDL, the lower the ratios of cholesterol to TG in HDL and LDL decreased to levels around the mean-2SD of normal subjects (1.8, 2.3, respectively), as previously reported[21]. In the right panel of sample B, TG contents of chylomicrons and VLDL did not increase, and the ratios of cholesterol to TG in HDL and LDL decreased slightly.

Discussion

In this study we analyzed serum lipids and apolipoproteins in fasting and postprandial periods among 24 young normolipidemic subjects. When we divided subjects into two groups according to the peak values of TG after loading, we found that the high TG group showed higher concentrations of TG, RemL-C, RLP-C, RLP-TG, and apoB-48 in the fasting period, although some parameters did not show significant differences. These concentrations were obviously elevated after loading in the high TG groups.

It is clear that an increase in chylomicrons and their remnants derived from the intestine is observed in the postprandial period; however, some investigators have reported that VLDL and their remnants derived from the liver are also increased, and it is



Sample A	0	1h	2h	4h	6h	8h
TC (mg/dL)	188	185	183	181	180	182
LDL-C (mg/dL)	124	118	113	111	115	117
HDL-C (mg/dL)	46	45	44	42	43	46
TG (mg/dL)	89	169	230	152	85	67

Sample B	0	1h	2h	4h	6h	8h
TC (mg/dL)	178	174	173	175	179	196
LDL-C (mg/dL)	92	88	88	94	91	101
HDL-C (mg/dL)	72	68	67	72	71	78
TG (mg/dL)	77	86	102	79	56	47

Fig. 2. Densitometric scanning electrophoretogram patterns of samples before and after loading the test meal. Elution profiles were analyzed by an automatic densitometer, Chol/Trig Combo™.

Sample A: Representative elution profile in the group with >150 mg/dL triglyceride (TG) concentration elevation after loading the test meal. Sample B: Representative elution profile in the group with <150 mg/dL TG concentration elevation before and after loading the test meal. HDL: high-density lipoproteins, VLDL: very-low-density lipoproteins, LDL: low-density lipoproteins, CM: chylomicrons, TG: triglyceride, red area: cholesterol, blue-colored area: TG.

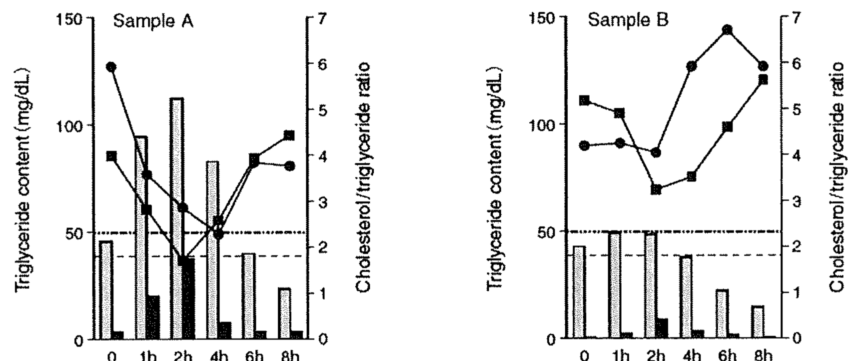


Fig. 3.

Changes in triglyceride (TG) levels of very-low-density lipoproteins (VLDL) and chylomicrons, and the ratios of cholesterol to TG of high-density lipoproteins (HDL) and low-density lipoproteins (LDL) before and after loading the test meal. Open column: TG content of VLDL, closed column: TG content of chylomicrons, closed circle: ratios of cholesterol to TG of HDL, closed square: ratio of cholesterol to TG of LDL, dashed line: ratio of cholesterol to TG of HDL of the mean-2SD in control subjects, dotted line: ratio of cholesterol to TG of LDL of the mean-2SD in control subjects.

controversial which is predominant in exogenous and endogenous lipids in the postprandial period[6, 22, 23]. Although the detailed mechanism remains unknown, the postprandial increase in VLDL and their remnants may be caused by reduced clearance, which was a result of competition by chylomicrons for the removal of triglycerides by lipoprotein lipase, or increased hepatic secretion of VLDL[22]. Receptor-mediated mechanisms are the predominant pathway by which chylomicron remnants are taken up by hepatocytes, and the LDL receptor pathway is thought to be the major mechanism for the uptake of both remnants with apoE as a ligand[12, 24, 25]. Impairment of or competition for the removal of both remnants in the liver may also potentially induce an increased retention time of these lipoproteins in the blood circulation. Thus, both remnant concentrations may be elevated in the postprandial period. In this study there was a remarkable elevation of apoB-48, which is a component of chylomicrons and chylomicron remnants, corroborating that lipoproteins derived from the intestine increase in the postprandial period, especially from 1 to 4 hours. ApoB concentrations (most derived from the liver) did not alter remarkably, suggesting that the postprandial increase in VLDL and their remnants may be small in young normolipidemic subjects.

Data of sample A (high TG group) demonstrated that delayed clearance of TG-rich lipoproteins in the postprandial period may be detected even in normolipidemic subjects. Impaired removal of TG-rich lipoproteins may induce the change of cholesterol and TG composition in LDL and HDL via the mechanism by which cholesteryl ester transfer protein is mediated[26, 27]; the higher the TG concentrations of chylomicrons and VLDL, the lower the ratios of cholesterol to TG of HDL and LDL. Thus, our data suggest that it may be possible to characterize lipid profiles conveniently in the fasting period by measuring TG, RemL-C, RLP-C, RLP-TG, and apoB-48 concentrations to detect postprandial hyperlipidemic subjects.

In the postprandial period, the elevation of RemL-C concentration did not reach a significant level, although the elevation period of RemL-C is similar to those of TG, RLP-C, and RLP-TG. These results may be due to the different measuring methods. RLP-C and RLP-TG were measured by the immune adsorption method[17], while RemL-C is measured by a newly developed and convenient assay for remnant lipoproteins[18]. This assay utilizes surfactant and phospholipase-D to directly solubilize and degrade remnants. As such, it can be performed with an automated clinical analyzer in a short time[18]. There was reportedly a strong correlation between RemL-C and RLP-C concentrations in patients with coronary artery disease[28]; however, our results (**Table 1**) suggest that differences in sensitivity for exogenous and endogenous lipoproteins between both methods may exist. The method for RLP-C and RLP-TG may be more sensitive to exogenous remnants, while RemL-C may be suitable for endogenous remnants. This hypothesis is compatible with previous reports[18, 28]. When we compared the two groups, the different character between RLP-C and RemL-C became clear (**Table 2** and **Fig. 1**). The high TG group had significantly higher fasting TG and RemL-C concentrations. After loading, RLP-C, RLP-TG, and apoB-48 also became significant parameters. It can be deduced that, in the fasting period, exogenous remnants in postprandial hyperlipidemic subjects may decrease to a similar level to that in normal subjects, and endogeneous remnants may remain at a significantly higher level.

There are some limitations of the present study as follows: as the study was performed with a small number of normal young subjects, only 4 individuals had the peak value of TG over 150 mg/dL as the high group. We need further examination with a larger number of normal and hyperlipidemic subjects in order to verify the TG value of 150 mg/dL, to identify the most dangerous lipid profile(s) involving remnants, apoB-48, and other parameters, and to clarify the significant differences between RLP-C and RemL-C. In addition, analyses of apoCs in remnant lipoproteins and LPL are necessary because these enzyme and proteins interfere with the apoE-mediated uptake of remnants and lipolysis[29, 30]. Here, since we did not examine LPL activity and protein mass measurement, we could not determine whether subjects in the high TG group have heterozygous LPL deficiency[31]. Of note, fasting sd-LDL-C concentration was higher in the high TG group. Interestingly, sd-LDL-C concentrations gradually decreased after loading. Here, we have no data to explain why sd-LDL-C in the high TG group but not in the low TG group gradually decreased after loading. Recently, Ogita *et al.* have reported that serum sd-LDL-C concentrations decreased after the 75 g oral glucose tolerance test and suggested that insulin can be a key modulator of sd-LDL-C concentrations[32].

In conclusion, TG, RLP-C, RLP-TG, RemL-C, and apoB-48 concentrations in the fasting period may be suitable to detect and characterize postprandial hyperlipidemia in normolipidemic subjects. In future, it is necessary to reveal which parameter or combination is useful to identify postprandial hyperlipidemia with a large-scale study.

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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

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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

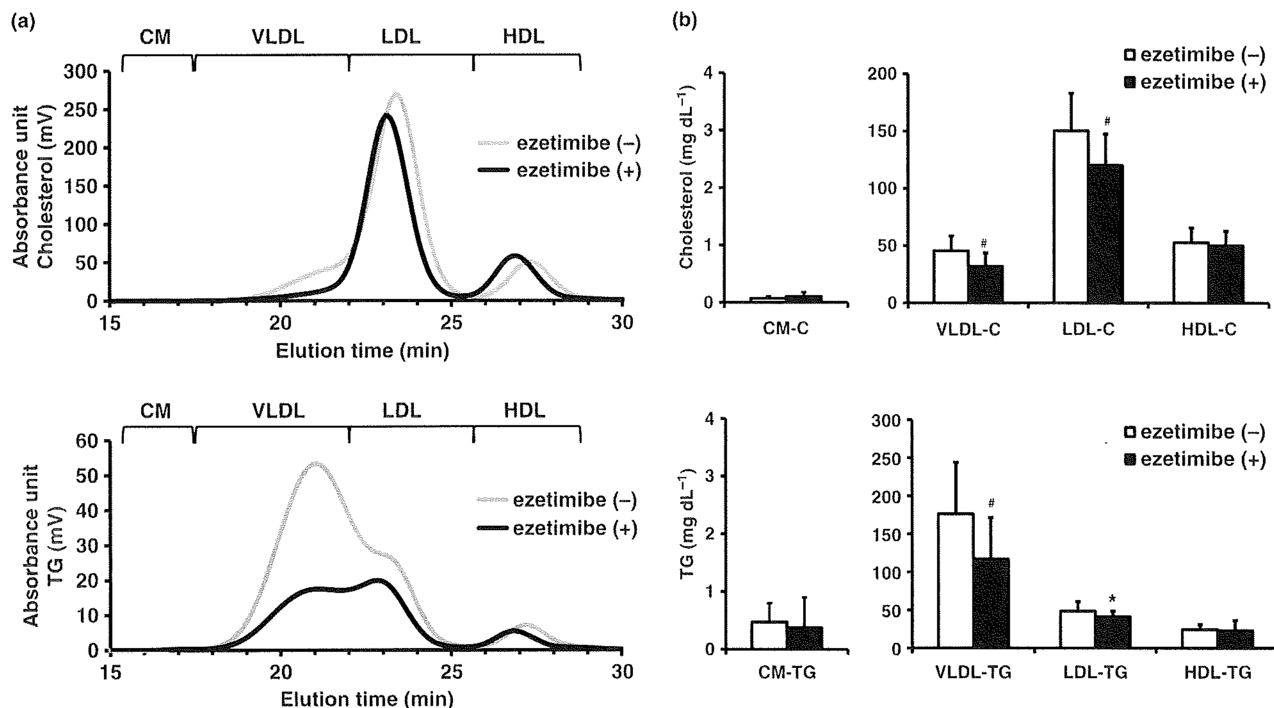

Figure 1 Lipoprotein profiles in the fasting state before and after administration of ezetimibe. Ezetimibe 10 mg was administered in patients with type IIb hyperlipidaemia ($n = 10$, two females and eight males) for 2 months. Two hundred microlitres of serum were separated from blood drawn in the fasting 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. $*P < 0{\cdot}05$, $\#P < 0{\cdot}005$.

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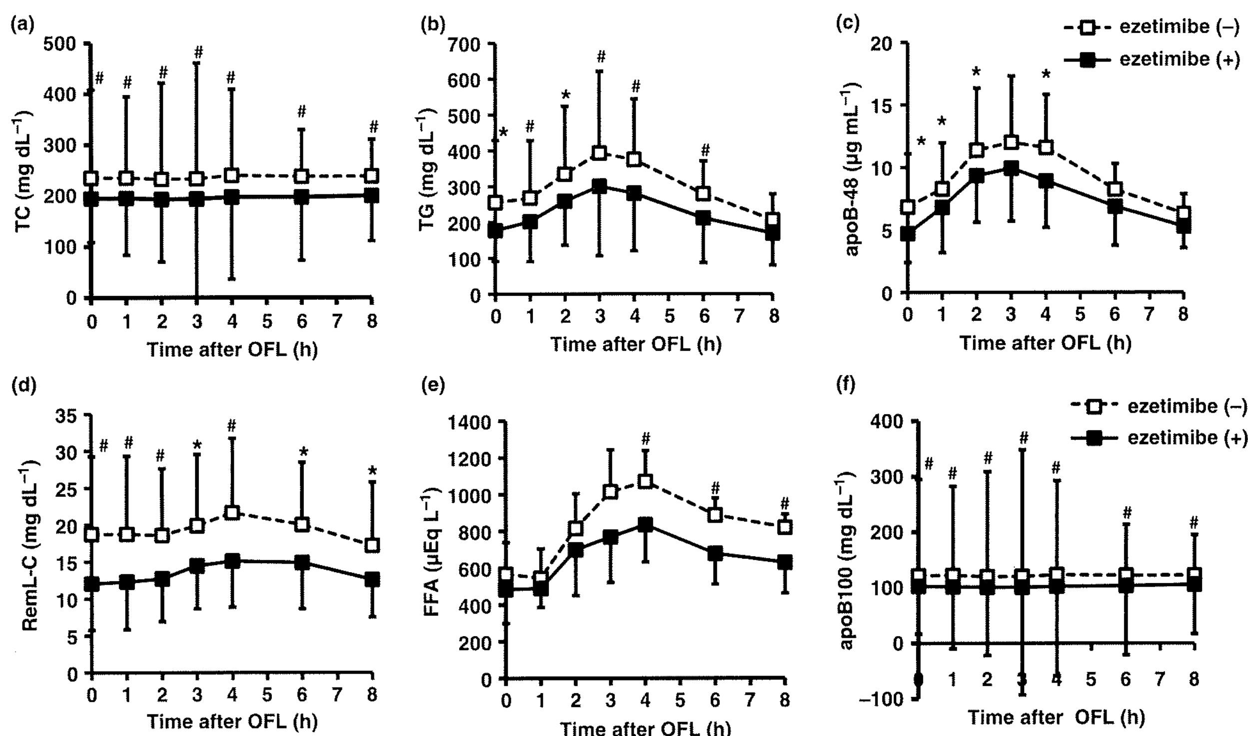

Figure 2 Oral fat loading (OFL) test before and after administration of ezetimibe. Patients with type IIb hyperlipidaemia (n = 10, two females and eight males) were given OFTT cream (containing 35% fat without sugar, 30 g fat m^{-2} body surface area) after overnight fasting before (open squares) and 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

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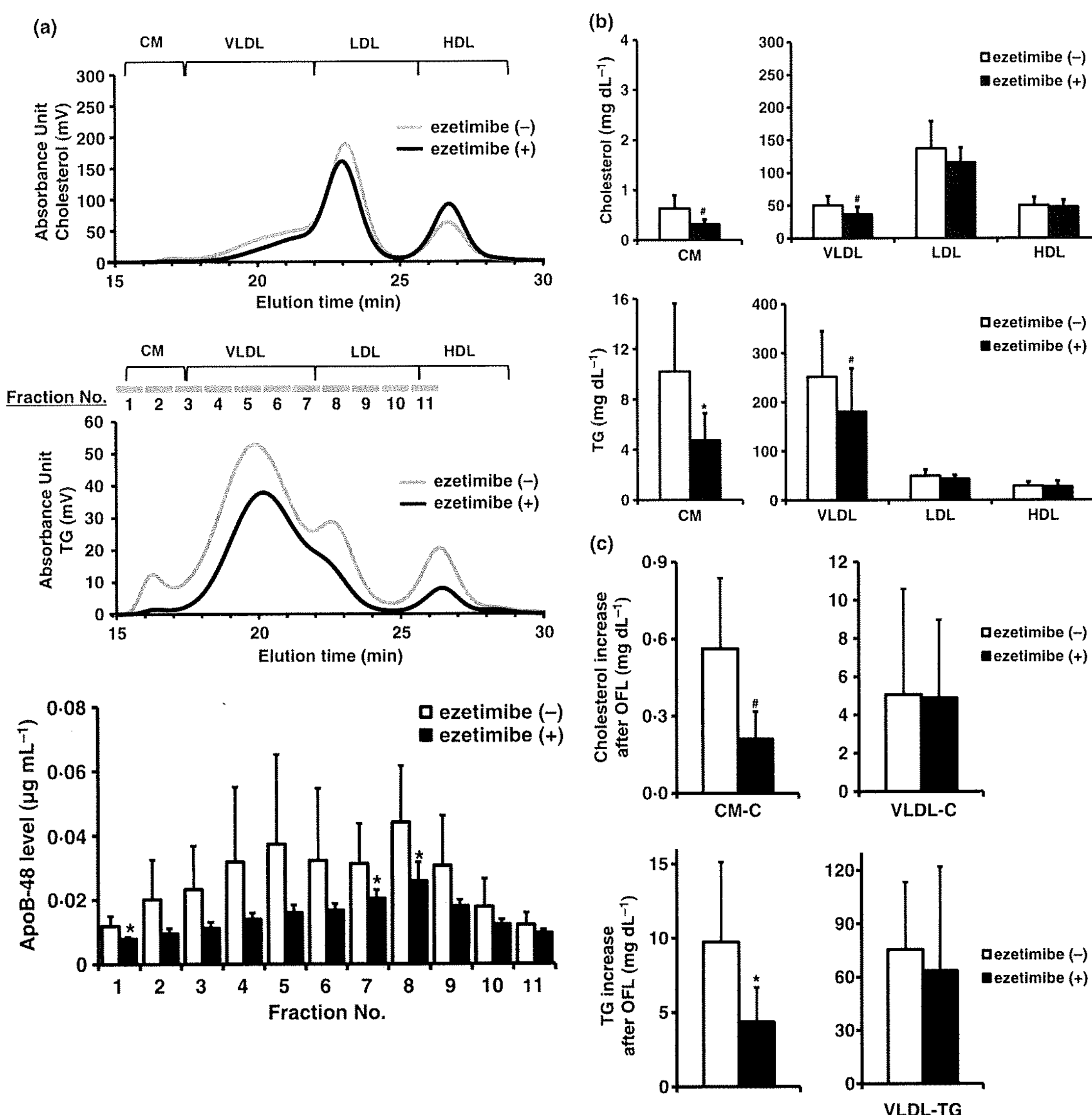

Figure 3 Lipoprotein profiles in postprandial state and incremental fasting/postprandial serum cholesterol and triglyceride (TG) levels before and after administration of ezetimibe. Two hundred microlitres of serum were separated from blood samples drawn 4 h after oral fat loading (OFL) before (open 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

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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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