

ORIGINAL

# Detrimental effects of high-fat diet loading on vascular endothelial function and therapeutic efficacy of ezetimibe and statins in patients with type 2 diabetes

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**Abstract.** Several recent reports from large clinical trials have described the role of postprandial hyperlipidemia in the onset of atherosclerosis. In this pilot study, the effects of postprandial lipid abnormalities induced by high-fat diet loading on vascular endothelial function in type 2 diabetes were investigated and the effects of ezetimibe and statins on endothelial function were compared. In 20 patients in Study 1, peripheral arterial tonometry tests were performed before and 4h after loading to measure the reactive hyperemia index (RHI). In Study 2, the same patients were randomly allocated to ezetimibe or rosuvastatin. After 1 week of treatment, loading tests were conducted in the same manner. In Study 1, the RHI decreased from 1.86 to 1.60. There were no significant correlations between changes in RHI and the area under the curve (AUC) or coefficient of variation (CV) of each metabolic marker. In Study 2, ezetimibe treatment resulted in a significant improvement in RHI. The two drugs had comparable effects on changes in AUC. There were no significant correlations between changes in RHI and changes in AUC or changes in CV. When age, sex, drug, hemoglobin A1c, and changes in each lipid were evaluated as independent variables with RHI improvement as the dependent variable, drug differences were found to exert the greatest effect on RHI improvement using a stepwise procedure. The results of this study suggest that the progression of atherosclerosis is due to abnormalities in postprandial lipid metabolism and that ezetimibe can potentially inhibit the aggravation of vascular endothelial dysfunction after high-fat diet loading.

**Key words:** Postprandial hyperlipidemia, Atherosclerosis, Type 2 diabetes, Reactive hyperemia index (RHI), Ezetimibe

**THE JAPAN DIABETES COMPLICATIONS STUDY (JDCS)** that investigated Japanese patients with type 2 diabetes identified low-density lipoprotein (LDL) hypercholesterolemia and hypertriglyceridemia to have the strongest influence on coronary artery disease, after adjustment for age and sex [1]. The Prospective Cardiovascular Münster (PROCAM) Study showed that low level of high-density lipoprotein (HDL)-C is an independent predictive factor

for cardiovascular disease even in patients with low LDL-C level, and that the higher the triglyceride (TG) level (within a range of <800 mg/dL), the more frequent the occurrence of major coronary events [2]. This emphasizes the need to control HDL hypocholesterolemia and hypertriglyceridemia. Previous studies that examined the relationship between non-fasting TG levels and the onset of atherosclerotic disease demonstrated significant correlations between TG levels and risks of cardiovascular disease, sudden death [3], and stroke [4]; indicating that postprandial hyperlipidemia plays a role in the development of atherosclerotic diseases. Postprandial hyperlipidemia is partially due to the accumulation of TG-rich lipoproteins, especially chylomicrons and chylomicron remnants derived from the intestine. Increased levels of apolipoprotein B-48,

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which is a constituent of chylomicrons and chylomicron remnants, significantly correlated with the morbidity of atherosclerotic cardiovascular disease [5].

Statins are the most frequently used drugs for the treatment of lipid abnormalities affecting vascular endothelial function. The Collaborative Atorvastatin Diabetes (CARDS) study [6] provided clear evidence for the usefulness of statin therapy in preventing the onset of macrovascular pathologies in type 2 diabetic patients lacking history of macrovascular disorders. However, statin therapy is associated with certain risks. For example, in the Treating to New Targets (TNT) study [7], high-dose statin therapy reduced the relative risk of major cardiovascular events by 22% in comparison with standard statin therapy, but the study also showed that the remaining 78% of cases required intervention by other agents. The reason for that finding was postulated to be that inhibition of cholesterol synthesis by statin therapy induced compensatory enhancement of cholesterol absorption, thereby elevating blood concentrations of the drug. In studies on the effects of lipid-lowering drugs on vascular endothelial function, 4-week treatment of patients with congestive heart failure (CHF) with 10 mg of rosuvastatin resulted in a significant improvement of flow-mediated vasodilatation (FMD) compared with 20 mg ezetimibe, demonstrating the usefulness of statin therapy in patients with heart disease [8]. Another study showed that 4-week administration of 10 mg ezetimibe in 10 healthy subjects achieved significant inhibition of decrease in FMD after high-fat diet loading in comparison with the control (no ezetimibe), suggesting that ezetimibe influences vascular endothelial function [9]. However, only a few studies have compared the effects of statins and ezetimibe on vascular endothelial function.

The outcome of FMD has been assessed in several studies and the results of FMD have been compared with those of invasive techniques that measure epicardial vascular function. In comparison, peripheral arterial tonometry (Endo-PAT) has low interobserver and intraobserver variability and less than ideal correlation with indices of microvascular function measured by invasive procedures [10]. The aim of the present study was to determine the effects of postprandial lipid abnormalities induced high-fat diet loading on vascular endothelial function and the effects of ezetimibe and statins on endothelial function tested by Endo-PAT, which is approved as the only non-invasive vascular endothelial function test by Food and Drug Administration (FDA)]

in diabetic patients with vasculopathies.

## Subjects and Methods

### Subjects

The research participants were 20 in patients with type 2 diabetic patients, aged between 20 and 79 years, who were not being treated for dyslipidemia, at the University of Occupational and Environmental Health, Department of Endocrinology, Metabolism and Diabetes and affiliated hospitals between April 2012 and March 2014. We excluded patients treated with insulin, and those with abnormalities in the electro-cardiogram. Although there were no restrictions on the use of oral antihyperglycemic agents at the time of admission, change of drugs was prohibited until the end of the study. The Institutional Review Board of the University of Occupational and Environmental Health approved this study. This Clinical Trial was registered with the University Hospital Medical Information Network (UMIN) (No. UMIN 000018577, 000018629). The study was explained to participants in writing, and written consent was obtained. Samples were processed appropriately according to the Declaration of Helsinki.

### Study design

Study 1 was a cross-sectional study in which the high-fat diet loading test was conducted in fasting patients in the morning of the second day of hospital stay. Blood samples were collected before high-fat diet loading as well as 1, 2, 3 and 4 h after loading, and TG, LDL-C, HDL-C, malondialdehyde modified low-density lipoprotein (MDA LDL-C), small-dense LDL-C (sd LDL-C), free-fatty acids (FFA), remnant like particles cholesterol (RLP-C), PG and apoB-48 were measured. The Endo-PAT test was performed before and 4 h after high-fat diet loading to measure the reactive hyperemia index (RHI), a marker of vascular endothelial function.

In Study 2, which was a randomized controlled study; subjects were allocated randomly by the envelope method to either of two treatment groups: ezetimibe 10 mg/day or rosuvastatin 2.5 mg/day. After 1-week oral administration of the allocated drug, the high-fat diet-loading test was conducted in the same manner as in Study 1.

The primary endpoint of Study 1 was changes in RHI before and after high-fat diet loading, and the secondary endpoint was the correlation between changes

in each lipid profile indicator and changes in RHI. In Study 2, the primary endpoint was the difference between RHI changes following treatment with each of the two drugs, and the secondary endpoint was the difference between changes in lipid and glucose metabolism markers in the two groups.

### **Biochemical analyses**

Blood samples were collected early in the morning after at least 12 h fasting, through a venous line placed in the median vein using an indwelling catheter. The PG level was measured with the glucose oxidase method. Hemoglobin A1c (HbA1c) was measured by high-performance liquid chromatography (HPLC) using Yosoh HLC-723 G8 (Tosoh Co., Kyoto, Japan). HbA1c (%) was estimated as the National Glycohemoglobin Standardization Program (NGSP) equivalent value, which was calculated as HbA1c (NGSP) (%) = HbA1c (JDS) (%) + 0.4%, considering the relationship of HbA1c (NGSP) values to HbA1c (JDS) (%) values measured by the Japanese standard and measurement method. The homeostasis model assessment for insulin resistance (HOMA-IR), which represents insulin resistance, was calculated (formula:  $\text{HOMA-IR} = \text{Fasting glucose level} \times \text{Fasting Insulin Level} \div 405$ ). Blood samples were collected during fasting and urinary C-peptide reactivity (u-CPR) levels were measured in 24 h urine samples.

Measurement of lipid profiles and other markers was outsourced to SRL Co., Ltd. Plasma lipid was measured with a Hitachi 7350 autoanalyzer (Hitachi Co., Tokyo). LDL-C was measured using the colestest LDL (Sekisui Medical, Tokyo) by the direct method. HDL-C was measured using the colestest NHDL (Sekisui Medical, Tokyo) by the direct method. TG was measured using the pureanto STG-N (Sekisui Medical, Tokyo) by the enzymatic method. FFA was measured using the NEFA-SS“EIKEN” (Eiken Kagaku, Tokyo) by the enzymatic method. sd LDL-C was measured using the sd LDL-EX reagent “SEIKEN” (Denka Seiken Inc., Tokyo) by the enzymatic method. MDA LDL-C was measured using the oxidative ELISA “Daiichi” (Sekisui Medical, Tokyo) by a sandwich ELISA (enzyme linked immunosorbent assay) method. RLP-C was measured using the RLP-C reagent “JIRO-II” (Otsuka Inc., Tokyo) by the immunoaffinity isolation method. ApoB-48 was measured using a chemiluminescence enzyme immunoassay (CLEIA, Fuji Rebio Inc., Tokyo) [11]. All samples

were stored at  $-80^{\circ}\text{C}$  until measurement.

### **Assessment of endothelial function with Endo-PAT**

We assessed vascular function in all 20 patients by Endo PAT 2000. The method used for digital assessment of vascular function using PAT has been described in detail previously [12]. Briefly, after 30 min acclimatization period in a room controlled for temperature and light in the fasting state, the baseline pulse amplitude was recorded during a period of 5 min before the induction of ischemia, which was induced by placing a blood pressure cuff on the upper arm, while the opposite arm served as a control. The PAT probes were placed on one finger of each hand. After 5 min, the blood pressure cuff was inflated to 60 mmHg above the systolic pressure or 200 mmHg for 5 min and then deflated to induce reactive hyperemia. As a measure of reactive hyperemia, RHI was calculated as the ratio of the average amplitude of the PAT signal over 1 min beginning 1.5 min after cuff deflation (control arm, A; tested arm, C) divided by the average amplitude of the PAT signal over the 2.5 min time period before cuff inflation (baseline) (control arm, B; tested arm, D). Thus,  $\text{RHI} = (\text{C/D}) / (\text{A/B}) \times \text{baseline correction}$ . Because RHI has a heteroscedastic error structure, we used a natural logarithm transformation in all analyses.

### **Oral fat loading test (OFLT)**

The high-fat diet-loading test was conducted in the morning after 12 h overnight fasting. The high-fat diet contained a total of 928 kcal, 58.6 g lipid (57% of the total calories), 68.3 g carbohydrates (30% of the total calories), and 31.1 g protein (13% of the total calories). The ingredients were similar to those of an American fast-food meal (Big Mac<sup>®</sup> with French fries, Orange juice<sup>®</sup>). Patients were asked to eat this high-fat and high-glucose meal (cake *s  le*) within 20 min. Blood samples were collected during the fasting state and 1, 2, 3 and 4 h after the load. In all patients, the OFLT was performed under stable conditions.

### **Statistical analysis**

Data were expressed as mean  $\pm$  standard deviation (SD). Normality was determined by the Shapiro-Wilk test. Values of TG, FFA, RLP-C, sd LDL-C, PG, apoB-48 showed skewed distribution. For one-sample comparison, the paired *t*-test was used for parameters with normal distribution, whereas the Wilcoxon test was used for parameters with skewed distribution. The

two sample *t*-test was used for normally distributed data and Mann-Whitney U test was used for data with skewed distribution. Regarding time-course changes in each metabolic marker in Study 1, preprandial and postprandial values were compared by the Bonferroni *t*-test. With regard to univariate analysis, we used Pearson correlation for normally distributed data and Spearman rank correlation for variables with skewed distribution. Multivariate stepwise regression analysis was conducted using RHI as the dependent variable and several parameters found to be significantly related to RHI on univariate analysis. We calculated AUC by using trapezoidal method. The level of significance was set as  $P < 0.05$ . All Statistical analyses were conducted using The Statistical Package for Social association version 21.0 (SPSS Inc., Chicago, IL).

## Results

### Clinical characteristics

The demographic details are shown in Table 1.

Of the 20 participants, 10 were males and 10 were females. The mean age of participants was  $54.4 \pm 11.3$  years. Participants were mildly obese, with mean BMI of  $24.8 \pm 5.2$  kg/m<sup>2</sup>. Blood glucose was generally poorly controlled on admission, with mean HbA1c levels at  $10.3 \pm 1.4\%$ . In addition, participants were insulin resistant on average, with a mean HOMA-IR of  $2.4 \pm 1.7$  and u-CPR level of  $72.0 \pm 47.0$  µg/day. The LDL-C level was  $114.4 \pm 38.9$  mg/dL, the HDL-C level was  $44.0 \pm 12.7$  mg/dL, and the TG level was  $166.4 \pm 97.8$  mg/dL, showing hypertriglyceridemia. TG level was  $\geq 150$  mg/dL in 10 (50%) of the 20 subjects. Table 2 shows the demographic details of patients of the ezetimibe and rosuvastatin groups in Study 2. There was no significant difference in all parameters between the two groups.

### High fat load test Study 1

As shown in Fig. 1, high-fat diet loading resulted in increased serum levels of TG, RLP-C, FFA, PG,

**Table 1** Baseline characteristics of all patients, ezetimibe group and rosuvastatin group

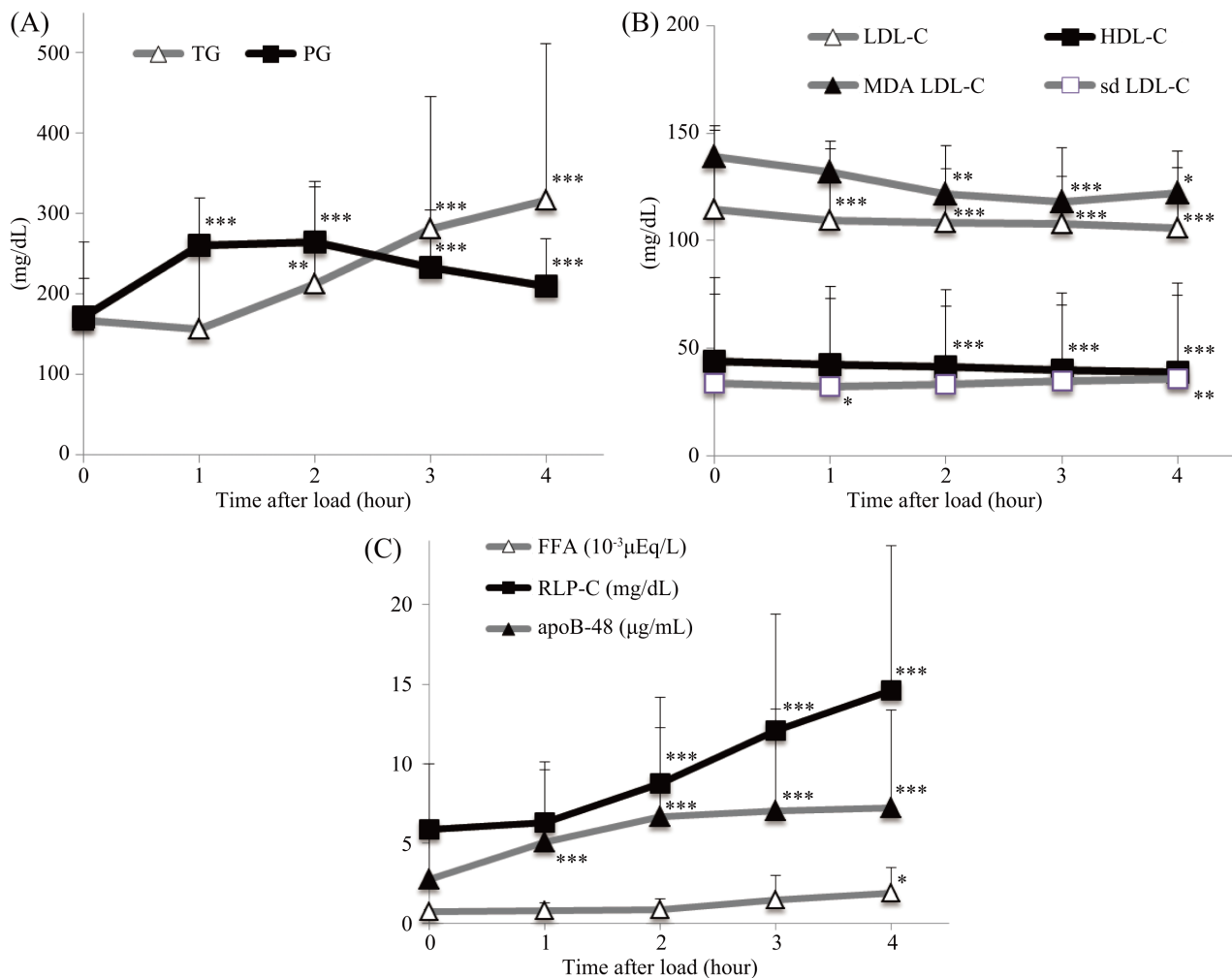
	All patients (10:10)	Ezetimibe group (5:5)	Rosuvastatin group (5:5)	<i>p</i> value
Sex (male: female)	(10:10)	(5:5)	(5:5)	>0.999
Age, years	$54.4 \pm 11.3$ (30-71)	$51.2 \pm 10.9$	$57.5 \pm 11.4$	0.272
Body weight, kg	$64.8 \pm 14.2$ (48.3-94.4)	$69.8 \pm 14.1$	$59.9 \pm 13.3$	0.096
Body mass index, kg/m <sup>2</sup>	$24.8 \pm 5.2$ (17.8-40.1)	$26.6 \pm 6.6$	$23.1 \pm 2.7$	0.162
Duration of diabetes, years	$6.8 \pm 7.9$ (1-23)	$6.7 \pm 8.8$	$6.8 \pm 7.3$	0.639
Diabetic neuropathy, n (%)	15 (75%)	6 (60%)	9 (90%)	0.121
Diabetic retinopathy, n (%)	6 (30%)	2 (20%)	4 (40%)	0.329
Diabetic nephropathy, n (%)	5 (25%)	2 (20%)	3 (30%)	0.606
HbA1c, %	$10.3 \pm 1.4$ (8.4-12.8)	$10.4 \pm 1.6$	$10.1 \pm 1.1$	0.791
Fasting plasma glucose, mg/dL	$171 \pm 47.9$ (117-269)	$186 \pm 53.9$	$156 \pm 38.2$	0.150
Insulin, µU/mL	$6.4 \pm 3.9$ (2.3-16.9)	$5.9 \pm 3.6$	$4.8 \pm 2.4$	0.496
HOMA-IR	$2.4 \pm 1.7$ (0.5-6.8)	$2.9 \pm 2.1$	$1.9 \pm 1.2$	0.364
u-C peptide, µg/day	$72.0 \pm 47.0$ (11.5-214.0)	$61.7 \pm 27.2$	$83.5 \pm 62.2$	0.567
TG, mg/dL	$166 \pm 98$ (67.0-423.0)	$182 \pm 134$	$151 \pm 39.7$	0.650
LDL-C, mg/dL	$114 \pm 38.9$ (61.0-218.0)	$116 \pm 52$	$113 \pm 21.9$	0.650
HDL-C, mg/dL	$44.0 \pm 12.7$ (21.0-76.0)	$44.9 \pm 15.2$	$43.1 \pm 10.4$	0.623
RLP-C, mg/dL	$5.9 \pm 4.1$	$7.3 \pm 5.3$	$4.6 \pm 1.5$	0.677
apoB-48, µg/mL	$2.73 \pm 2.72$	$1.64 \pm 1.90$	$3.82 \pm 3.06$	0.140

Data are mean±SD (range: minimum-maximum) or n (%). *P* values indicate the difference between the ezetimibe and rosuvastatin groups, by two-sample *t*-test for normally distributed data and by Mann-Whitney U test for data with skewed distribution. Diabetic neuropathy, retinopathy and nephropathy were valued by chi-square test. HbA1c, hemoglobin A1c; HOMA-IR, homeostasis model assessment insulin resistance; TG, triglyceride; LDL, low-density lipoprotein; HDL, high-density lipoprotein; RLP-C, Remnant Like Particles Cholesterol; apoB, apolipoprotein B.

**Table 2** Area under the curve (AUC) of various parameters of lipid and glucose metabolism, change in AUC for ezetimibe and rosuvastatin groups

	Ezetimibe group			Rosuvastatin group			<i>p</i> value
	Before	After	Change in AUC	Before	After	Change in AUC	
TG AUC (mg/dL·h)	1011 ± 670	497 ± 273	-514 ± 464	771 ± 263	581 ± 143	-190 ± 295	0.212
LDL-C AUC (mg/dL·h)	443 ± 194	334 ± 108	-109 ± 109	430 ± 82	263 ± 80	-166 ± 102	0.238
HDL-C AUC (mg/dL·h)	166 ± 56	155 ± 36	-11.7 ± 26.6	163 ± 37	156 ± 35	-7.6 ± 23.9	0.721
MDA LDL-C AUC (mg/dL·h)	508 ± 151	395 ± 165	-113 ± 98	498 ± 160	332 ± 104	-166 ± 193	0.449
sd LDL-C AUC (mg/dL·h)	150 ± 76	80.3 ± 28.5	-69.5 ± 61.1	119 ± 36	70.0 ± 23.5	-49.0 ± 32.1	0.762
FFA AUC (μEq/L·h)	4665 ± 4265	3325 ± 2404	-1340 ± 3018	4088 ± 2590	3137 ± 1620	-951 ± 1437	0.940
RLP-C AUC (mg/dL·h)	45.7 ± 28.5	18.2 ± 7.7	-27.5 ± 25.7	29.2 ± 10.8	19.5 ± 6.5	-9.7 ± 12.6	0.151
apoB-48 AUC (μg/mL·h)	18.9 ± 16.9	11.1 ± 10.6	-7.8 ± 16.3	28.9 ± 23.1	18.4 ± 15.7	-10.4 ± 11.6	0.406
PG AUC (mg/dL·h)	1012 ± 263	649 ± 110	-363 ± 242	884 ± 203	721 ± 148	-163 ± 154	0.059

Data are mean±SD. *P* values represent the difference between the ezetimibe and rosuvastatin groups, by two-sample *t*-test for normally distributed data and by Mann-Whitney U test for data with skewed distribution. AUC was calculated by the trapezoidal method. Abbreviations as in Table 1, MDA LDL-C, malondialdehyde modified low-density lipoprotein; sd LDL-C, small dense low-density lipoprotein; FFA, free fatty acid; PG, plasma glucose.

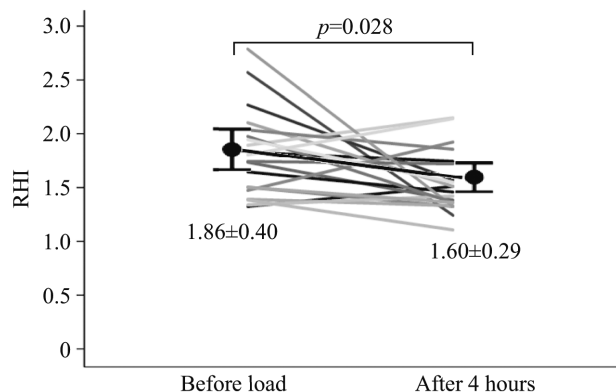
**Fig. 1** Serum levels of various metabolic parameters measured before high-fat load and 4 h after the load. (A) TG, PG, (B) LDL-C, HDL-C, MDA LDL-C, sd LDL-C, (C) FFA, RLP-C, apoB-48. *P* values indicate the difference between fasting stage and each time point, by Bonferroni *t*-test. \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001, vs. 0 h.



and apoB-48 and reduced the levels of LDL-C, HDL-C, sdLDL-C, and MDA LDL-C. Fig. 2 shows the effects of high-fat diet loading on changes in vascular endothelial function. The RHI value was  $1.86 \pm 0.40$  before high-fat diet loading, but decreased significantly to  $1.60 \pm 0.29$  4 h after loading ( $p=0.028$ ). The AUC of each metabolic marker and the coefficient of variation (CV) were examined in relation to changes in RHI. Assessment of apoB-48, AUC of each metabolic marker, and the CV value showed that fasting apoB-48 correlated positively with TG AUC ( $r=0.468$ ,  $p=0.037$ ) and TG CV ( $r=0.446$ ,  $p=0.049$ ). Moreover, apoB-48 AUC correlated with TG AUC ( $r=0.575$ ,  $p=0.008$ ), TG CV ( $r=0.482$ ,  $p=0.031$ ), and RLP-C CV ( $r=0.482$ ,  $p=0.031$ ), but not with other parameters (data not shown).

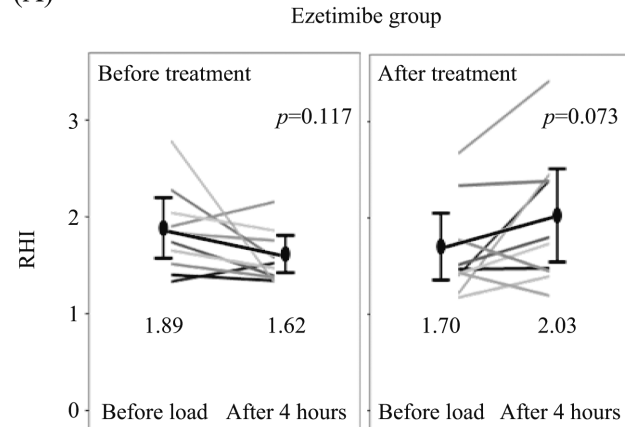
## Study 2

As shown in Fig. 3, RHI tended to decrease in both the ezetimibe and rosuvastatin groups before drug administration, similar to Study 1 (ezetimibe group, 1.89 to 1.62,  $p=0.117$ , rosuvastatin group, 1.83 to 1.58,  $p=0.157$ , respectively) (Fig. 3A, B). However, after 1 week of treatment, RHI still showed a decreasing trend in the rosuvastatin group (1.87 to 1.54,  $p=0.050$ ) (Fig. 3B), whereas it increased in the ezetimibe group (1.70 to 2.03,  $p=0.073$ ) (Fig. 3A). With regard to changes in RHI based on the administered drug, ezetimibe resulted in a significant improvement in RHI compared with rosuvastatin ( $p=0.014$ ) (Fig. 3C). On the other hand, there were no significant correlations between changes in RHI and changes in AUC (a marker of AUC improvement), and between changes in RHI and

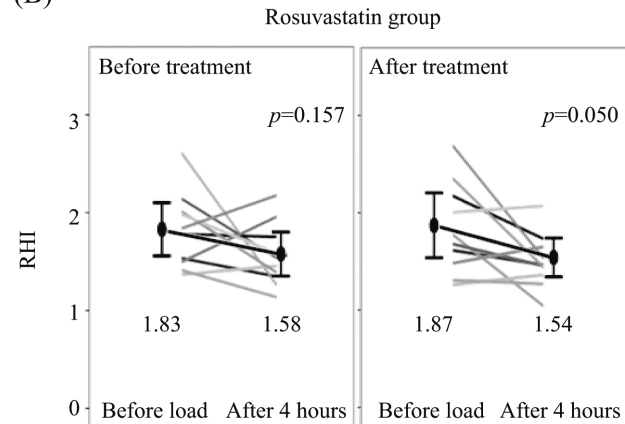


**Fig. 2** Differences in changes in RHI between before high-fat load and 4 h after high-fat load. *P* values indicate the difference between before load and 4 h after load, by the paired *t*-test.

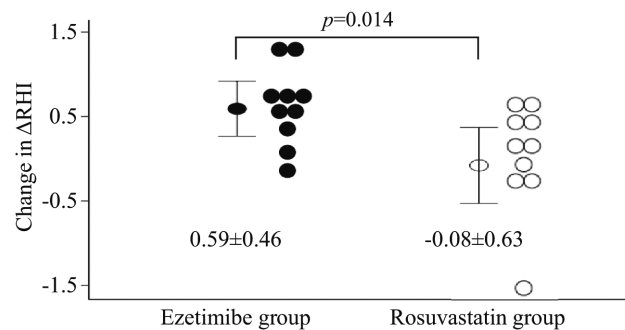
(A)



(B)



(C)



**Fig. 3** Differences in changes in RHI between before high-fat load and 4 h after high-fat load. (A) the ezetimibe group (B) the rosuvastatin group. *P* values indicate the difference between before load and 4 h after load, by paired *t* test. (C) Comparison of changes in  $\Delta$ RHI in the ezetimibe group and rosuvastatin group. *P* values indicate the difference between the two groups, by the paired *t*-test.

changes in CV (a marker of CV improvement). Table 2 shows changes in AUC according to the administered drug. The effects of the two drugs on changes in AUC were similar irrespective of the parameters examined. When age, sex, drug, hemoglobin A1c, and changes in each lipid parameter were examined as independent variables with improvement in RHI as the dependent variable, drug difference was found to have the largest influence on improvement in RHI in all subjects, using the stepwise procedure (adjusted multiple  $R^2=0.431$ , standardized coefficient  $\beta=-0.542$ ,  $p=0.006$ ).

## Discussion

In Study 1, high-fat diet loading reduced vascular endothelial function in patients with type 2 diabetes, suggesting that the existence of postprandial hyperlipidemia may facilitate the progression of endothelial dysfunction. Several clinical studies have previously investigated the lipid profile and the effects of dietary load on vascular endothelial function. In healthy adult volunteers, a cookie load (75 g carbohydrate, 28.5 g fat and 8 g protein, total 592 kcal (SARAYA Corp, Osaka, Japan) decreased FMD in a manner that correlated significantly with variations in TG and apolipoprotein B-48 (apoB-48) [9]. On the other hand, we showed in patients with type 2 diabetes that the diet loading test (using Test meal A: total 450 kcal; carbohydrate 51.4%, fat 33.3%, protein 15.3%, a recipe proposed by a working group of the Japan Diabetes Society) resulted in a significant decrease in vascular endothelial function, which neither correlated with plasma glucose (PG) AUC ( $r=-0.475$ ,  $p=0.074$ ) nor with immunoreactive insulin (IRI) AUC ( $r=0.093$ ,  $p=0.742$ ), but with TG AUC ( $r=-0.780$ ,  $p=0.001$ ) [13]. Although the direct relation between vascular endothelial function and abnormalities of lipid metabolism was not investigated in this study, the results demonstrated that increased remnants due to fat loading correlated with decreased vascular endothelial function. Other factors (in addition to remnants) that aggravate vascular endothelial dysfunction, such oxysterols (oxidized derivatives of cholesterol) and oxidative stress, were not investigated in this study, and could be involved. Because the increase of remnants in postprandial hyperlipidemia causes inflammatory reactions through oxysterol and oxidative stress [14, 15], these reactions may also have a synergistic effect on aggravating vascular endothelial reaction. In previous studies in healthy subjects, mea-

surements up to 8 h after cookie loading (a total of 592 kcal with 28.5 g fat constituting 43.3% of the total calories) showed that FMD was lowest at 4 h, and changes in this parameter had a significant negative correlation with the maximum values of TG and apoB-48 (both TG and apoB-48 reached maximum values at 4 h) [9]. In the present study, the proportion of lipid was very high at 57%, and measurement was carried out only up to 4 h after loading. Therefore, the correlation between other metabolic markers and the observed vascular endothelial dysfunction could have been observed had the loading test been prolonged to determine the peak TG at a time point longer than 4 h after loading.

In this study, the target of measurement was apoB-48, a major constituent apoprotein of intestine-derived, exogenous lipoproteins (chylomicrons and chylomicron remnants). Because only one molecule of apoB-48 is present in each lipoprotein particle, it can be a quantitative marker that represents chylomicron and chylomicron remnants. Its blood level in the fasting state is useful for screening postprandial hyperlipidemia [16]. According to the report by Tanimura *et al.* [17], high fasting apoB-48 levels are present in diabetic patients with carotid artery plaques; TG incremental AUC and fasting apoB-48 correlated significantly after high-fat diet-loading in 10 healthy men. The present study investigated the correlation between apoB-48 level and vascular endothelial function, but no direct correlation was found between changes in RHI (which reflect vascular endothelial function) and apoB-48 AUC. However, fasting apoB-48 levels correlated with TG AUC and TG CV and apoB-48 AUC correlated with TG AUC, TG CV, and RLP-C CV. Thus, apoB-48 levels correlated with apoB-48 AUC under the condition of postprandial hyperlipidemia. These findings are consistent with those of previous studies [16, 17], suggesting the usefulness of apoB-48 as an index of postprandial hyperlipidemia.

The results of Study 2 suggested that ezetimibe can improve RHI after high-fat loading to a significantly greater extent than rosuvastatin. However, analysis of parameters that could affect the improvement of RHI in the ezetimibe group showed no significant correlation with improvement in blood glucose levels, AUC of lipid metabolic markers or CV values. In a long-term study that compared the effects of statins and ezetimibe on vascular endothelial function in CHF patients, 10 mg/day simvastatin and 10 mg/day ezetimibe had similar effects on LDL-C, but the improvement in FDD

(flow-dependent dilation) was significantly greater with simvastatin, in part through inhibition of oxidative stress [18]. In another study, 4-week administration of 10 mg/day rosuvastatin in CHF patients achieved significantly better improvement in FMD than 20 mg/day ezetimibe [19]. There is general agreement that statins are more effective in improving vascular endothelial function in patients with heart disease, such as coronary artery disease and CHF. On the other hand, 4-week administration of 10 mg/day ezetimibe in healthy individuals was reported to improve FMD to a significantly greater extent than 10 mg/day pravastatin [20]. With respect to the effects on vascular endothelial function after high-fat diet-loading, the decrease in FMD after cookie loading was inhibited to a significantly greater extent in healthy subjects treated with 10 mg/day ezetimibe for 4 weeks than the control group [9]. However, there are no reports that compare ezetimibe and statin monotherapies in patients with type 2 diabetes tested under high-fat diet-loading. Thus, the present study is the first to suggest the higher efficacy of ezetimibe relative to statin in improving vascular endothelial function after high-fat diet loading in patients with type 2 diabetic patients.

Ezetimibe-mediated improvement of vascular endothelial function is presumed to be a pleiotropic effect. The pleiotropic effects of ezetimibe have been reported to include also the inhibition of high-sensitivity C-reactive protein and interleukin-6 in patients with lipid abnormalities associated with obesity [21], inhibition of monocyte chemoattractant protein-1 in diabetic patients with lipid abnormalities [17], inhibition of urinary 8-hydroxydeoxyguanosine in patients with chronic kidney disease and lipid abnormalities [22], decrease in reactive oxygen species in ApoE knockout mice [23], and facilitation of endothelial nitric oxide synthase production [24]. It has also been reported that 4-week treatment with 10 mg/day ezetimibe or 10 mg/day pravastatin had comparable positive effects on LDL-C, but only ezetimibe caused a decrease in Rho kinase activity in leukocytes, achieving improvement in FMD [20]. In our Study 2, the drug difference was clearest on RHI, suggesting that ezetimibe directly improved vascular endothelial function, independent of its lipid-improving action.

A recent large-scale clinical study (IMPROVE-IT) examined the inhibitory effects of the combination therapy of ezetimibe and statin on cardiovascular events [25]. The results showed that the combination therapy

significantly inhibited the occurrence of cardiovascular events more effectively than simvastatin monotherapy [25]. Further analysis in the same study showed no significant differences between diabetic patients and non-diabetic patients in relation to age, sex, presence/absence of lipid abnormalities at baseline, or LDL-C level; however, the combined use of ezetimibe and a statin led to a significantly greater inhibition of cardiovascular events in diabetic patients than in non-diabetic patients. Our results may add support to the findings of the above study and suggest that ezetimibe can induce stabilization of vascular endothelial function in diabetic patients through inhibition of remnants.

The present study has several limitations. First, the study was an open-label study with a small sample size; therefore, selection bias might have been involved. Further investigation in a large number of patients will be required. Second, the study did not examine the direct effects of ezetimibe on vascular endothelial function, including inflammatory cytokines, adhesion factors, and oxidative stress mediators. Interrelated factors for improvement of postprandial vascular endothelial function in this study were not identified, and any direct effect of ezetimibe on vascular endothelial function that was not investigated in this study cannot be ruled out. Ezetimibe is reported to inhibit disorders of vascular endothelial function by suppressing oxidative stress and inflammation. The results of this pilot study in a small number of type 2 diabetic patients suggest that 1-week treatment with ezetimibe improves vascular endothelial function-based RHI. Further long-term studies of larger sample size are necessary to determine the cellular and molecular mechanisms of such improvement, especially the effects of longer treatment with ezetimibe on inflammatory cytokines, adhesion factors, and mediators of oxidative stress.

In summary, study 1 showed that high-fat diet-loading decreased vascular endothelial function in patients with type 2 diabetes, suggesting that it is due to the progression of atherosclerosis, at least in part, derived from abnormalities in postprandial lipid metabolism. Also study 2 showed that ezetimibe can potentially inhibit aggravation of vascular endothelial function after high-fat diet loading.

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## Disclosure

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## References

1. Sone H, Tanaka S, Tanaka S, Iimuro S, Oida K, et al. (2011) Japan Diabetes Complications Study Group: Serum level of triglycerides is a potent risk factor comparable to LDL cholesterol for coronary heart disease in Japanese patients with type 2 diabetes: subanalysis of the Japan Diabetes Complications Study (JDCS). *J Clin Endocrinol Metab* 96: 3448-3456.
2. Assmann G, Schulte H, Cullen P, Seedorf U (2007) Assessing risk of myocardial infarction and stroke: new data from the Prospective Cardiovascular Münster (PROCAM) study. *Eur J Clin Invest* 37: 925-932.
3. Iso H, Naito Y, Sato S, Kitamura A, Okamura T, et al. (2001) Serum triglycerides and risk of coronary heart disease among Japanese men and women. *Am J Epidemiol* 153: 490-499.
4. Freiberg JJ, Tybjaerg-Hansen A, Jensen JS, Nordestgaard BG (2008) Nonfasting triglycerides and risk of ischemic stroke in the general population. *JAMA* 300: 2142-2152.
5. Masuda D, Sugimoto T, Tsujii K, Inagaki M, Nakatani K, et al. (2012) Correlation of fasting serum apolipoprotein B-48 with coronary artery disease prevalence. *Eur J Clin Invest* 42: 992-999.
6. Colhoun HM, Betteridge DJ, Durrington PN, Hitman GA, Neil HA, et al. (2004) CARDS investigators: Primary prevention of cardiovascular disease with atorvastatin in type 2 diabetes in the Collaborative Atorvastatin Diabetes Study (CARDS): multicentre randomised placebo-controlled trial. *Lancet* 364: 685-696.
7. LaRosa JC, Grundy SM, Waters DD, Shear C, Barter P, et al. (2005) Treating to New Targets (TNT) Investigators: Intensive lipid lowering with atorvastatin in patients with stable coronary disease. *N Engl J Med* 352: 1425-1435.
8. Gounari P, Tousoulis D, Antoniadou C, Kampoli AM, Stougiannos P, et al. (2010) Rosuvastatin but not ezetimibe improves endothelial function in patients with heart failure, by mechanisms independent of lipid lowering. *Int J Cardiol* 142: 87-91.
9. Yunoki K, Nakamura K, Miyoshi T, Enko K, Kohno K, et al. (2011) Ezetimibe improves postprandial hyperlipidemia and its induced endothelial dysfunction. *Atherosclerosis* 217: 486-491.
10. Flammer AJ, Anderson T, Celermajer DS, Creager MA, Deanfield J, et al. (2012) The assessment of endothelial function: from research into clinical practice. *Circulation* 126: 753-767.
11. Sakai N, Uchida Y, Ohashi K, Hibuse T, Saika Y, et al. (2003) Measurement of fasting serum apoB-48 levels in normolipidemic and hyperlipidemic subjects by ELISA. *J Lipid Res* 44: 1256-1262.
12. Bonetti PO, Pumper GM, Higano ST, Holmes DR Jr, Kuvin JT, et al. (2004) Noninvasive identification of patients with early coronary atherosclerosis by assessment of digital reactive hyperemia. *J Am Coll Cardiol* 44: 2137-2141.
13. Torimoto K, Okada Y, Mori H, Otsuka T, Kawaguchi M, et al. (2015) Effects of exenatide on postprandial vascular endothelial dysfunction in type 2 diabetes mellitus. *Cardiovasc Diabetol* 14: 25.
14. Napolitano M, Bravo E (2005) Lipid metabolism and TNF-alpha secretion in response to dietary sterols in human monocyte derived macrophages. *Eur J Clin Invest* 35: 482-490.
15. Wang L, Sapuri-Butti AR, Aung HH, Parikh AN, Rutledge JC (2008) Triglyceride-rich lipoprotein lipolysis increases aggregation of endothelial cell membrane microdomains and produces reactive oxygen species. *Am J Physiol Heart Circ Physiol* 295: H237-244.
16. Masuda D, Sakai N, Sugimoto T, Kitazume-Taneike R, Yamashita T, et al. (2011) Fasting serum apolipoprotein B-48 can be a marker of postprandial hyperlipidemia. *J Atheroscler Thromb* 18: 1062-1070.
17. Tanimura K, Nakajima Y, Nagao M, Ishizaki A, Kano T, et al. (2008) Association of serum apolipoprotein B-48 level with the presence of carotid plaque in type 2 diabetes mellitus. *Diabetes Res Clin Pract* 81: 338-344.
18. Landmesser U, Bahlmann F, Mueller M, Spiekermann S, Kirchhoff N, et al. (2005) Simvastatin versus ezetimibe: pleiotropic and lipid-lowering effects on endothelial function in humans. *Circulation* 111: 2356-2363.
19. Gounari P, Tousoulis D, Antoniadou C, Kampoli AM, Stougiannos P, et al. (2010) Rosuvastatin but not ezetimibe improves endothelial function in patients with heart failure, by mechanisms independent of lipid lowering. *Int J Cardiol* 142: 87-91.

20. Sugimura K, Fukumoto Y, Satoh K, Nochioka K, Miura Y, et al. (2012) Percutaneous transluminal pulmonary angioplasty markedly improves pulmonary hemodynamics and long-term prognosis in patients with chronic thromboembolic pulmonary hypertension. *Circ J* 76: 485-488.
21. Chan DC, Watts GF, Gan SK, Ooi EM, Barrett PH (2010) Effect of ezetimibe on hepatic fat, inflammatory markers, and apolipoprotein B-100 kinetics in insulin-resistant obese subjects on a weight loss diet. *Diabetes Care* 33: 1134-1139.
22. Nakamura T, Sato E, Fujiwara N, Kawagoe Y, Ueda Y, et al. (2009) Ezetimibe decreases serum levels of asymmetric dimethylarginine (ADMA) and ameliorates renal injury in non-diabetic chronic kidney disease patients in a cholesterol-independent manner. *Pharmacol Res* 60: 525-528.
23. Nakagami H, Osako MK, Takami Y, Hanayama R, Koriyama H, et al. (2009) Vascular protective effects of ezetimibe in ApoE-deficient mice. *Atherosclerosis* 203: 51-58.
24. Kuhlencordt PJ, Padmapriya P, Rützel S, Schödel J, Hu K, et al. (2009) Ezetimibe potently reduces vascular inflammation and arteriosclerosis in eNOS-deficient ApoE ko mice. *Atherosclerosis* 202: 48-57.
25. Cannon CP, Blazing MA, Giugliano RP, McCagg A, White JA, et al. (2015) IMPROVE-IT Investigators: Ezetimibe added to statin therapy after acute coronary syndromes. *N Engl J Med* 372: 2387-2397.