TABLE 2. Plasma Concentrations, FCRs, and PRs of VLDL-apoB, IDL-apoB, and LDL-apoB From HD Patients and Control Subjects

Subjects	VLDL-apoB, mg/dl	VLDL-apoB-FCR, days^{-1}	VLDL-apoB-PR, mg/kg/day	IDL-apoB, mg/dl	IDL-apoB-FCR, days^{-1}	IDL-apoB-PR, mg/kg/day	LDL-apoB, mg/dl	LDL-apoB-FCR, days^{-1}	LDL-apoB-PR, mg/kg/day
Controls									
1	4.0	8.47	16.17	n.d.	n.d.	n.d.	48.0	0.242	5.54
2	1.6	24.49	16.81	n.d.	n.d.	n.d.	186.2	0.314	25.12
3	5.2	6.09	13.02	n.d.	n.d.	n.d.	111.6	0.582	26.69
4	0.3	4.39	0.51	n.d.	n.d.	n.d.	41.7	0.563	10.50
5	1.2	8.45	4.24	n.d.	n.d.	n.d.	111.0	0.280	13.33
6	13.5	15.88	90.71	n.d.	n.d.	n.d.	78.4	0.211	7.01
7	3.0	43.56	56.07	n.d.	n.d.	n.d.	143.0	0.929	57.01
8	0.4	7.77	1.26	n.d.	n.d.	n.d.	95.3	0.596	22.99
9	1.5	34.13	21.68	n.d.	n.d.	n.d.	82.5	0.298	10.55
10	6.6	6.00	17.13	6.8	4.66	13.84	68.2	0.527	15.64
11	4.6	10.77	22.57	5.7	6.22	16.16	70.4	0.552	17.75
12	3.2	8.74	12.24	1.7	15.83	11.50	67.8	0.430	12.65
13	3.5	8.21	12.30	1.5	8.86	5.86	72.0	0.480	14.99
Patients									
1	14.5	7.48	53.76	n.d.	n.d.	n.d.	143.1	0.209	14.83
2	0.2	36.50	3.43	n.d.	n.d.	n.d.	92.7	0.199	9.15
3	0.8	10.35	4.15	n.d.	n.d.	n.d.	91.9	0.120	5.53
4	5.7	6.50	18.59	n.d.	n.d.	n.d.	74.2	0.175	6.50
5	5.4	4.35	10.92	n.d.	n.d.	n.d.	83.2	0.077	2.99
6	0.3	8.49	1.32	n.d.	n.d.	n.d.	134.2	0.210	14.64
7	8.2	10.05	41.32	n.d.	n.d.	n.d.	145.9	0.057	4.14
8	4.6	8.49	20.87	5.4	3.93	11.27	68.9	0.449	16.51
9	3.0	6.14	10.11	4.1	3.79	8.58	68.3	0.110	4.13
10	7.0	4.27	15.71	8.1	2.25	9.57	70.3	0.340	12.50
11	6.1	4.83	15.40	5.1	1.52	4.06	69.8	0.380	13.86
12	7.9	3.66	14.58	8.1	2.88	11.79	92.5	0.268	12.50
Controls, Mean±SD	3.7±3.5	14.38±12.18	21.90±24.91	3.9±2.7	8.89±4.94	11.84±4.42	90.5±39.8	0.462±0.197	18.44±13.30
Patients, Mean±SD	5.3±4.0	9.26±8.87	17.51±15.51	6.2±1.8	2.87±1.02	9.05±3.08	94.6±29.7	0.216±0.123	9.77±4.91
P Value	0.221	0.157	0.663	0.327	0.014	0.221	0.550	0.001	0.026

n.d. indicates not determined.
See Table 1 for patient and control subject specifications.

between normotensive HD patients and controls, which is the prerequisite for calculating PV using the formula of Mitra et al.[30] Previous investigations, however, have found very minor differences in the relative BV between HD patients and controls.[32,37] HD results in a relative BV reduction in the range of up to 15% per ultrafiltration cycle.[38] Even when suspecting a persistently reduced BV on the interdialytic day (which was our day of investigation), this effect is very unlikely having caused the large difference in PRs of LDL-apoB between patients and controls. The two other kinetic parameters (FCRs and residence times) are independent of parameters' blood concentrations.

Markers for malnutrition and inflammation are widely recognized as predictors for cardiovascular disease in chronic kidney disease.[39] Our HD patients did not show signs of inflammation or malnutrition: although their plasma albumin, total protein, and C-reactive protein plasma levels differed significantly from those of controls, they were within normal range. In fact, plasma levels in HD patients should be corrected for their Hct levels to be accurately comparable to those of healthy controls.[27] This calculation would result in even higher mean values for total protein and albumin in HD patients compared with controls. Resulting C-reactive protein values would then still be within normal range (except patient #4 whose kinetic parameters were, nevertheless, all close to the mean levels of the whole patient group).

A decreased FCR for IDL and LDL apoB is identical to an extended residence time of these highly atherogenic particles. The longer residence time of these lipoprotein fractions results in an extended oxidation time of IDL and LDL in a highly oxidative environment. This was in fact experimentally shown by a highly significant correlation of 5-hydroxy-2-aminovaleric acid (HAVA) in LDL, an oxidation product of apoB-100, with the LDL residence time in normolipidemic controls.[40] In line with these results, two recent randomized placebo-controlled studies revealed a significant reduction in

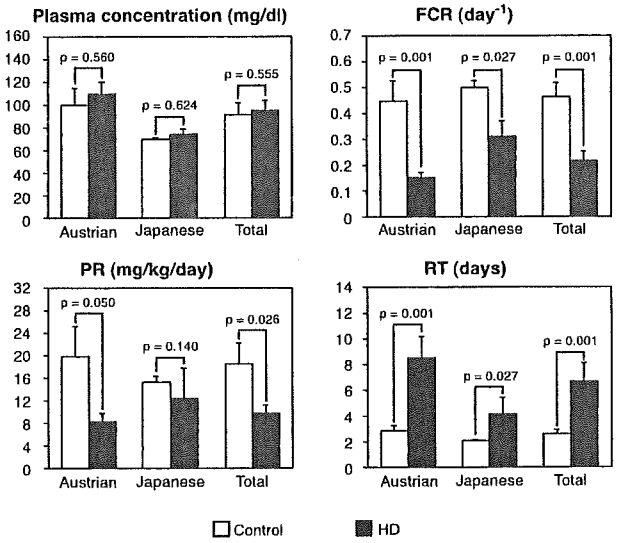

Figure 2. Kinetic parameters of apoB from LDL. Plasma concentrations of LDL-apoB as well as the respective FCR, residence time and PR values are indicated for HD patients (black columns) and controls (white columns). Bars show standard error of means. Values are expressed for the total study group as well as separately for Austrian and Japanese study groups.

composite cardiovascular disease end points when HD patients were treated for two years with supplementation of antioxidants like vitamin E[41] or acetylcysteine.[42]

Most remarkably, the observed substantially impaired metabolism of apoB-100–containing lipoproteins is accompanied by normal levels of LDL-apoB and elevated levels of IDL-apoB (Table 2, Figure 2), in line with previous reports which found increased levels of IDL as an independent risk factor for atherosclerosis in HD patients.[43]

Our kinetic data seem to contrast with a previously published turnover study in Finnish HD patients performed with conventional radiotracer techniques. Although the authors found decreased LDL clearance rates in predialysis patients with chronic kidney failure,[18] they could not find a significant difference in LDL-apoB FCR between their HD patients and controls.[17] The reason for this discrepancy is not clear. There might be ethnic differences in the lipoprotein metabolism between Finns, Austrians, and Japanese although we could not detect substantial differences in our data when stratified into Japanese and Austrian subgroups (Figure 2). FCR and residence time values of LDL-apoB differed significantly between HD patients and controls in both ethnic groups. The differences in PR of LDL-apoB between cases and controls did only reach significance when calculated in the total groups and showed only borderline significance when considered separately for each ethnic group, suggesting limited statistical power with respect to PR values. One major difference to the above-mentioned study is an age difference between patients and controls in our study but not in the Finnish study. Our control subjects were considerably younger than the HD patients (35 versus 51 years). At first glance, this age difference might explain to some extent the dramatic differences found in our study because LDL clearance rates have been repeatedly described to decrease with age presumably attributable to downregulated hepatic LDL receptor expression in the elderly.[44,45] Based on the results of these studies, an age difference of 15 years (as observed in our work) would result in an ≈10% change in FCR values and could not therefore explain the more than 2-fold difference in our study. Comparative analysis of an age-matched subsample of five patients (subjects 1, 3, 5, 10, and 11, mean age 38.8 years) and controls (subjects 2, 3, 4, 5, and 11, mean age 38.2 years) revealed very similar mean LDL-FCR values (0.225 and 0.458 pools/d, respectively) as compared with the whole study collective. In addition, LDL-apoB FCR did not correlate with age in our study (HD patients: $r = -0.112$, $P=0.728$; controls: $r=0.165$, $P=0.590$), no matter whether the total group or Japanese and Austrian subjects were calculated separately. The observed differences in kinetic parameters therefore cannot be explained by age differences between study groups. Finally, when we reanalyzed the data from the Finnish study, we found 2 HD patients (subjects 10 and 11) who had substantially higher LDL apoB FCR (0.451 and 0.472 pools/d) than did the remaining HD patients. Indeed, when compared without these 2 outliers, LDL apoB FCR was found also to be significantly decreased in the HD group (0.306 pools/day) as compared with the control group (0.376 pools/day, $P=0.0008$) indicating agreement with our results.

Several mechanisms might contribute to our observations. First, the diminished LDL catabolism in HD patients might be explained by a possible contribution of LDL uptake by the healthy human kidney, which does not function appropriately in chronic kidney failure. In fact, glomerular cells like mesangial or epithelial cells have been shown in vitro to express lipoprotein receptors and to take up LDL comparably to fibroblasts and hepatocytes.[46] It is, however, completely unclear whether the kidney plays a significant role in LDL catabolism in vivo. Perfusion studies in rat kidneys indicated that virtually no intact LDL is cleared from the circulation by the kidney.[47] Second, the impaired lipolytic cascade in HD patients most likely also contributes to our results. The relatively normal VLDL levels and kinetic parameters and the corresponding impaired IDL parameters are in good accordance with previous findings of normal lipoprotein lipase masses but significantly decreased activities of hepatic triglyceride lipase (HTGL) in HD patients.[48] Because HTGL promotes the conversion of IDL to LDL, a decrease in HTGL activity might contribute to the accumulation of IDL and reduced PRs of LDL (without accumulating small dense LDL) in HD patients. In fact, analysis of the Japanese subjects of this study showed the conversion rate from IDL to LDL (k[5,4]) to be significantly decreased by 68% to 2.87 ± 1.02 pools per day in HD patients as compared with 8.89 ± 4.94 pools per day in control subjects ($P=0.014$), which is consistent with the previously reported 47% decrease in HTGL activity in HD patients.[48]

Disorders in the metabolism of LDL with normal circulating plasma LDL levels have been reported to result from overproduction and increased clearance of LDL (reviewed by Grundy et al[49]). Several impairments in LDL metabolism, including reduced clearance and increased PRs, have been described in various renal diseases.[17,18,50] In contrast to the

results shown in this study in HD patients, they all, however, result in elevated LDL plasma levels. Because lipoprotein metabolism substantially differs between the various stages and treatment modalities of chronic kidney disease (reviewed in[5]), it is not surprising to find the respective kinetic parameters of VLDL-, IDL-, LDL-apoB differently reported between predialysis, HD, and peritoneal dialysis.[17–19]

The implications of our study are far-reaching and not restricted to the investigated patient group. To the best of our knowledge, this is the first described example of a clinical condition in which reduced synthesis and clearance rates of an atherogenic lipoprotein are masked by normal plasma concentrations. The obtained results therefore demonstrate the need for in vivo kinetic studies to understand complex metabolic systems in humans, even in situations where the snap-shot ex vivo values seem to be almost normal. In particular, the observed alterations in lipoprotein metabolism put HD patients at high risk for developing atherosclerotic disease despite their normal cholesterol and LDL cholesterol plasma levels. These patients should therefore be identified and given appropriate therapy. In fact, recent studies using lipid-lowering therapy of ESRD (including HD) demonstrated a substantial normalization of the dyslipidemic plasma profile and reduced progression of renal disease[51,52] and in one study also showed reduced mortality[53] in these patients. Because most lipid-lowering drugs act by "normalizing" the residential times of the major atherogenic lipoproteins IDL and LDL,[54] these drugs are expected to correct some of the basic defects of the severely disturbed lipoprotein metabolism in HD patients. Kinetic studies of the impact of lipid-lowering medication on the lipoprotein metabolism of ESRD (including HD) patients are therefore urgently required.

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Effects of diacylglycerol ingestion on postprandial hyperlipidemia in diabetes

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Abstract

Background: We previously reported that diacylglycerol (DAG) as compared with triacylglycerol (TAG) suppressed increases in postprandial lipids in healthy volunteers. This study was to investigate the effects of DAG on postprandial lipids, particularly remnant lipoproteins in diabetics.

Methods: Emulsified DAG oil or TAG oil with a fatty acid composition similar to DAG oil was orally administered (30 g fat/m^2 of body surface) to moderately controlled six diabetics, with hemoglobin A1c (HbA1c) below 8%, after fasting for at least 12 h in a randomized crossover manner. Serum cholesterol and TAG, lipids in remnant-like particles (RLP), and other lipid parameters including serum ketone bodies were measured prior to and 2, 4, and 6 h after fat loading.

Results: DAG loading significantly suppressed increases in postprandial serum TAG and lipids in RLP as compared with TAG loading. The incremental area under the curve (IAUC) for serum TAG and that for lipids in RLP with DAG loading were also significantly smaller than those with TAG loading. However, changes in serum levels of insulin, free fatty acids, and ketone bodies during fat loading were essentially the same for DAG and TAG.

Conclusions: This pilot study suggests that substituting DAG intake for TAG may be beneficial to moderately controlled diabetics due to its effect in reducing postprandial hyperlipidemia.

Keywords: Diacylglycerol; Remnant lipoprotein; Remnant-like particles; Postprandial hyperlipidemia; Diabetes

1. Introduction

A greatly enhanced risk for coronary heart disease (CHD) has been reported in diabetes [1,2]. Lipid abnormalities in diabetes, including elevated plasma tri-

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acylglycerol (TAG), low cholesterol in high-density lipoproteins (HDL), an increase in small, dense low-density lipoproteins (LDL), and postprandial hyperlipidemia contribute to this increased risk [3–6]. Among them, postprandial increase in remnant lipoproteins (remnants) has been recognized as a powerful CHD risk not only for diabetic but also for nondiabetic subjects [7–12].

Remnants are the metabolites of TAG-rich lipoproteins, such as chylomicrons (CM) and very-low-density lipoproteins (VLDL), and are formed in the circulation by the effect of lipoprotein lipase. These remnants are readily incorporated into endothelial macrophages, leading to the accumulation of cholesterol in these cells, consequently forming a premature atherosclerotic lesion [9,12–14].

Therapeutic approaches to reduce remnant levels in the postprandial phase are believed to be important for the management of patients with diabetes and also with metabolic syndrome [5]. We have previously reported [15] that the substitution of diacylglycerol (DAG) oil intake for TAG oil significantly suppressed postprandial increases in serum TAG and lipids in remnants measured by the method of Nakajima et al. [16] in healthy male volunteers. Therefore, we had an interest whether this favorable effect of DAG intake on postprandial hyperlipidemia can be applied to diabetic subjects without any serious adverse phenomenon.

Diacylglycerol is a natural component of various edible oils and consists mainly of the 1,3-species. The intake of DAG has been reported to reduce fasting serum TAG concentration and hemoglobin A1c (HbA1c) levels in type 2 diabetics and to prevent the accumulation of body fat in experimental animals and in humans [17,18]. Decreased activities of enzymes of fatty acid synthesis and increased activities of enzymes involved in the β-oxidation pathway by DAG ingestion have also been reported [18].

The objectives of this study were to investigate the effects of oral DAG loading on postprandial changes in serum lipids, related parameters including ketone bodies, and changes in remnants in moderately controlled diabetics.

2. Materials and methods

2.1. Subjects

The subjects were six patients with type 2 diabetes mellitus (five females and one male; aged 46–70 years) who had moderately controlled HbA1c levels that were <8%. The study was performed in accordance with the principle of the Helsinki Declaration. The subjects were fully informed concerning the study and gave their informed consent. The clinical characteristics of subjects are shown in Table 1. All of them were not receiving insulin therapy, but they were medicated as follows: one was taking both a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor and a hypotensive drug; five subjects were taking oral antidiabetic drugs (sulfonylurea, biguanides, and α-glucosidase inhibitors), and two of these five were also taking HMG-CoA reductase inhibitors, and the others were taking hypotensive drugs concomitantly.

Table 1
Characterics of subjects

No.	Age (years)	Height (m)	Weight (kg)	FPG (mmol/l)	HbA1c (%)	Serum TAG (mmol/l)	Serum T-Chol (mmol/l)	HDL-C (mmol/l)
1	59	1.47	59	7.3	6.5	1.75	4.85	2.12
2	46	1.50	65	7.5	7.8	0.89	5.35	1.27
3	70	1.48	54	6.1	6.5	0.69	5.53	1.32
4	67	1.64	57	11.5	7.6	1.34	5.06	1.11
5	59	1.47	47	9.3	7.6	0.88	5.47	2.24
6	68	1.57	56	9.6	6.8	0.59	5.64	1.86
Mean±S.E.	62±4	1.52±0.03	56±2	8.6±0.8	7.1±0.2	1.03±0.18	5.32±0.12	1.65±0.20

TAG: triacylglycerol; BMI: body mass index; FPG: fasting plasma glucose; T-Chol: total cholesterol.

2.2. Experimental oils

The DAG oil was prepared by esterifying glycerol with fatty acids from soybean and rapeseed oil according to the method of Huge-Jensen et al. [19]. The product contained 1,3-DAG and 1,2-DAG isomers in a ratio of 7:3 with a total DAG content of approximately ≥80%. Triacylglycerol oil was prepared by mixing rapeseed, safflower, and perilla oils to give a final fatty acid composition that was similar to that of the DAG oil. The fatty acid compositions of the DAG and TAG oils are shown in Table 2. No appreciable differences in combustion energy were detected between the DAG and TAG oils [20].

2.3. Study design

The study was designed in a double-blind cross-over style with a 2-week interval. The medications were not changed during the period of interval. The protocol was substantially the same as that as previously reported [15]. Briefly, the patients ingested emulsified test oil (TAG or DAG oil) at a dose of 30 g fat/m^2 of body surface area in the morning after fasting for approximately 12 h. The test oil emulsions contained 35% oil, 1% casein sodium, 3% skim milk, 0.5% fatty acid sucrose polyester, 0.36% soybean lecithin, and 60.14% water. During the study, patients were asked to remain seated with minimum physical activity. Patients were also requested not to take any drugs or food except for water from the morning of the test day to the final blood sample collection.

Blood samples were collected before fat loading for baseline measurements (initial value) and at 2, 4, and 6 h after fat loading. Two weeks later, the same patients received the opposite test oil, and blood samples were collected at the same time points.

Table 2
Fatty acid composition of test oils (wt.%)

	DAG	TAG
C16	3.1	5.4
C18	1.1	2.1
C18:1	38.3	34.3
C18:2	47.7	49.2
C18:3	9.0	7.8
C20	0.3	0.5
C20:1	0.2	0.3

DAG: diacylglycerol; TAG: triacylglycerol.

2.4. Sample analysis

Serum lipids, lipids in lipoproteins, HDL-cholesterol (HDL-C), and serum apolipoprotein concentrations were measured as described elsewhere [15]. The concentrations of serum total ketone bodies [21] and plasma glucose [22] were measured by enzymatic methods. Serum insulin [23] and plasma plasminogen-activator inhibitor 1 (PAI-1) [24] were measured by enzyme immunoassay methods. Plasma preheparin lipoprotein lipase (LPL) protein mass was measured by the method of Kobayashi et al. [25]. Serum leptin was measured by radioimmunoassay [26]. The remnant-like particle (RLP) fraction was isolated from the serum of each sample by means of an immunoaffinity-mixed gel conjugated with anti-apoB-100 and anti-apoAI monoclonal antibodies [16].

Lipoprotein fractions were obtained from the serum sample by sequential preparative ultracentrifugation [27] using a Hitachi RP65T rotor. Chylomicrons (Svedberg floatation >400 lipoproteins) were isolated at 20,000 rpm for 30 min at 15 °C, and very-low-density lipoproteins (VLDL; d<1.006 g/ml) were isolated further at 40,000 rpm for 16 h at 4 °C. Then, serum density was adjusted by adding potassium bromide. LDL (1.006<d<1.063 g/ml) and HDL (1.063<d<1.21 g/ml) fractions were isolated sequentialy at 40,000 rpm for 20 and 40 h, respectively, at 4 °C. Separated LDL and HDL were dialyzed against 0.15 mol/l NaCl. TAG concentrations of the isolated lipoprotein fractions were determined by enzymatic methods.

2.5. Statistical analysis

Measured values and changes (Δ) from the initial values are presented as mean±standard error (S.E.). Statistical analyses were performed using SPSS (version 11.0: SPSS, Chicago IL). Differences between the groups in the time course changes from the initial values were analyzed using repeated-measures two-way ANOVA. The difference between the measured values during DAG and TAG loading test at each time point was assessed by a paired t-test. The incremental area under the curve (IAUC) of blood variables during 6 h after fat loading was calculated,

Table 3
Changes in serum concentrations of lipids

		Time after fat loading (h)				p value of ANOVA
		0	2	4	6	
T-Chol (mmol/l)	DAG	5.25±0.16 (0.00)	5.35±0.13 (0.08±0.08)	5.22±0.10 (−0.05±0.08)	5.28±0.13 (0.03±0.08)	p=0.330
	TAG	5.38±0.16 (0.00)	5.35±0.13 (−0.03±0.05)	5.33±0.13 (−0.05±0.05)	5.28±0.13 (−0.10±0.05)	
LDL-C (mmol/l)	DAG	3.28±0.23 (0.00)	3.34±0.26 (0.08±0.08)	3.23±0.23 (−0.05±0.05)	3.18±0.23 (−0.10±0.08)	p=0.617
	TAG	3.26±0.28 (0.00)	3.26±0.26 (0.00±0.05)	3.18±0.26 (−0.08±0.05)	3.18±0.28 (−0.08±0.05)	
HDL-C (mmol/l)	DAG	1.66±0.21 (0.00)	1.63±0.18 (−0.03±0.03)	1.60±0.18 (−0.08±0.05)	1.63±0.21 (0.00±0.03)	p=0.567
	TAG	1.63±0.18 (0.00)	1.66±0.18 (0.00±0.05)	1.58±0.18 (−0.08±0.03)	1.60±0.18 (−0.05±0.00)	
TAG (mmol/l)	DAG	0.94±0.19 (0.00)	1.46±0.33 (0.52±0.23)	1.59±0.42 (0.64±0.23)	1.41±0.26 (0.14±0.11)	p=0.005
	TAG	1.11±0.20 (0.00)	1.77±0.32 (0.68±0.27)	1.80±0.34 (0.69±0.18)	1.41±0.36 (0.32±0.17)	
FFA (mmol/l)	DAG	0.51±0.06 (0.00)	0.63±0.07 (0.11±0.04)	0.87±0.09 (0.36±0.07)	0.82±0.11 (0.31±0.09)	p=0.567
	TAG	0.59±0.08 (0.00)	0.75±0.11 (0.16±0.16)	0.79±0.06 (0.21±0.11)	0.85±0.13 (0.27±0.13)	
RLP-C (mmol/l)	DAG	0.11±0.01 (0.00)	0.17±0.03 (0.06±0.03)	0.17±0.04 (0.06±0.02)	0.13±0.02 (0.03±0.01)	p=0.021
	TAG	0.11±0.02 (0.00)	0.21±0.02 (0.10±0.02)	0.20±0.03 (0.09±0.02)	0.15±0.03 (0.05±0.02)	
RLP-TAG (mmol/l)	DAG	0.14±0.04 (0.00)	0.48±0.15 (0.34±0.14)	0.52±0.19 (0.38±0.14)	0.29±0.08 (0.15±0.05)	p=0.004
	TAG	0.15±0.04 (0.00)	0.67±0.19 (0.52±0.18)	0.59±0.13 (0.44±0.10)	0.41±0.13 (0.26±0.09)	

Values are Mean±S.E. Mean±S.E. changes from baseline are shown in parentheses (Δ). *Significantly different from TAG ingesition at the same time points by paired t-test: $p<0.05$. P values are calculated by repeated-measures two-way ANOVA (Δ). DAG: diacylglycerol; TAG: triacylglycerol; T-Chol: total cholesterol; FFA: free fatty acids.

and the differences between the treatment groups were assessed by a paired t-test. P values <0.05 were considered to be significant for all analyses.

3. Results

3.1. Changes in serum lipids and apolipoproteins

Table 3 shows changes in serum lipids, LDL-C, HDL-C, and RLP lipids. Serum total cholesterol, LDL-C, and HDL-C did not change during TAG or DAG loading. However, serum TAG increased, peaking at 4 h after the loading with either oil and decreased at 6 h. Increases (Δ: shown in the parentheses in Table 3) in serum TAG from the initial value were significantly smaller during DAG loading than those observed during TAG loading (p=0.005), as determined by two-way ANOVA. No significant difference in serum TAG levels after fat loading was observed between the TAG and DAG study groups at any time point. Serum FFA was increased during TAG or DAG loading, and changes in these values were not different significantly between the two study groups.

The RLP lipids, RLP-C and RLP-TAG, were increased, peaking at 2 or 4 h and then decreased toward the initial value as a function of time after the loading of either oil. However, increases in RLP-C

Table 4
Changes TAG in CM, VLDL, LDL, and HDL

		Time after fat loading (h)				p value of ANOVA
		0	2	4	6	
CM (mmol/l)	DAG	0.01±0.01 (0.00)	0.27±0.13 (0.25±0.13)	0.30±0.12 (0.29±0.12)	0.12±0.05 (0.10±0.04)	p=0.141
	TAG	0.02±0.01 (0.00)	0.37±0.16 (0.35±0.16)	0.31±0.06 (0.30±0.05)	0.17±0.07 (0.15±0.07)	
VLDL (mmol/l)	DAG	0.38±0.16 (0.00)	0.55±0.18 (0.17±0.06)	0.59±0.22 (0.20±0.06)	0.36±0.16 (−0.02±0.05)	p=0.796
	TAG	0.52±0.16 (0.00)	0.70±0.17 (0.17±0.07)	0.72±0.22 (0.20±0.09)	0.53±0.21 (0.01±0.07)	
LDL (mmol/l)	DAG	0.34±0.04 (0.00)	0.33±0.04 (0.00±0.01)	0.33±0.04 (0.00±0.01)	0.32±0.05 (−0.01±0.02)	p=0.097
	TAG	0.34±0.04 (0.00)	0.34±0.04 (0.00±0.00)	0.35±0.04 (0.00±0.00)	0.35±0.04 (0.01±0.01)	
HDL (mmol/l)	DAG	0.14±0.02 (0.00)	0.16±0.02 (0.02±0.01)	0.18±0.03 (0.04±0.02)	0.16±0.03 (0.01±0.01)	p=0.585
	TAG	0.17±0.01 (0.00)	0.19±0.01 (0.02±0.01)	0.21±0.02 (0.03±0.02)	0.18±0.02 (0.01±0.01)	

Values are Mean±S.E. Mean±S.E. changes from baseline are shown in parentheses (Δ). P values are calculated by repeated-measures two-way ANOVA (Δ). DAG: diacylglycerol; TAG: triacylglycerol.

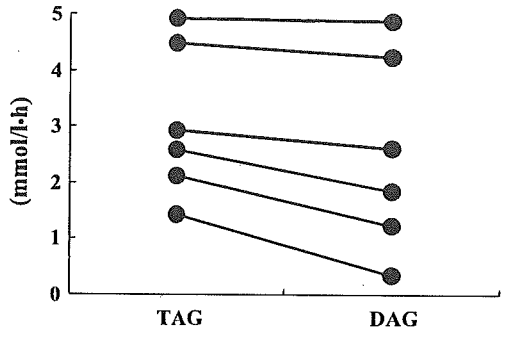

Fig. 1. Incremental area under the curve (IAUC) for serum TAG with either TAG or DAG loading (n=6). Increases in serum TAG were monitored during 6 h after a single loading of either TAG or DAG orally. This figure shows the difference of individual IAUC for serum TAG between TAG and DAG loading, and IAUC for serum TAG in the DAG group were significantly lower when compared with those in the TAG group (p<0.05). DAG: diacylglycerol; TAG: triacylglycerol.

and RLP-TAG from the initial values (Δ) after DAG loading were significantly smaller than those after TAG loading.

Changes in TAG level in each lipoprotein, separated by ultracentrifugation, with fat loading are shown in Table 4. No significant differences in terms of changes in these values were found between the TAG and DAG groups. However, the duration to the peak CM-TAG after fat loading was longer in the case of DAG loading compared with that in the TAG loading group, suggesting a slower production of CM during the DAG loading, although the difference between the groups was not significant. Apolipoproteins AI, AII, B, CII, CIII, and E were decreased during either fat loading, and changes in these parameters were not significantly different between TAG and DAG loading (data not shown).

3.2. Incremental area under the curve (IAUC) of serum TAG and RLP lipids

Differences of IAUC for serum TAG and RLP lipids between DAG and TAG ingestion, monitored up to 6 h after the loading with test oil, are shown in Figs. 1 and 2, respectively. The mean values of IAUC for serum TAG, RLP-C, and RLP-TAG in the DAG group were all significantly lower when compared with those in the TAG group (p<0.05).

3.3. Changes in serum insulin, plasma glucose, serum total ketone bodies, plasma LPL protein mass, serum leptin, and plasma PAI-1

As listed in Table 5, serum insulin was increased slightly at 2 h and then decreased at 4 and 6 h after either of the fat loading, and there were no significant differences between the values for TAG and DAG loading. Plasma glucose was gradually decreased with time after either of the fat loading. Decreases in plasma glucose from the initial value (Δ) at 2 and 4 h were significantly larger with TAG loading than those with DAG loading. Overall, decreases in plasma glucose during TAG loading were significantly greater

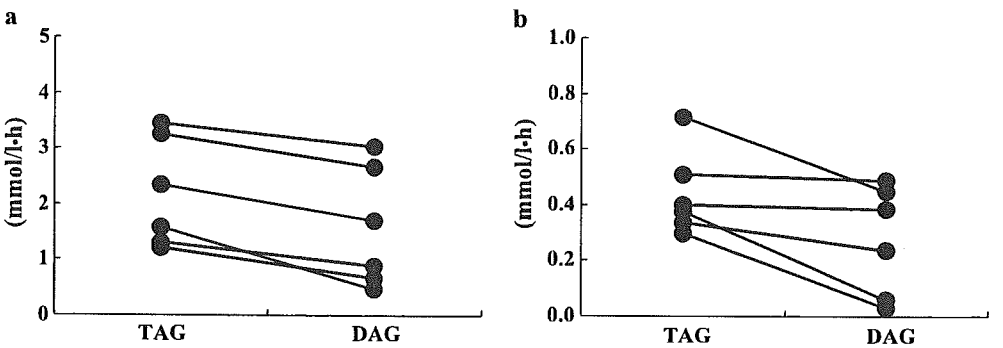

Fig. 2. Incremental area under the curve (IAUC) for RLP lipids with either TAG or DAG loading (n=6). Increases in RLP-TAG (a) and RLP-C (b) were monitored during 6 h after a single loading of either TAG or DAG orally. This figure shows the difference of individual IAUC for RLP-TAG (a) and RLP-C (b) between TAG and DAG loading. IAUC for both RLP-TAG (a) and RLP-C (b) in the DAG group were significantly lower when compared with those in the TAG group, respectively (each p<0.05). DAG: diacylglycerol; TAG: triacylglycerol; RLP: remnant-like particles.

Table 5
Changes in concentrations of serum insulin, plasma glucose, serum leptin, plasma LPL protein mass, plasma PAI-1

		Time after fat loading (h)				p value of ANOVA
		0	2	4	6	
Insulin (pmol/l)	DAG	45.0±13.2 (0.0)	46.8±6.6 (1.8±9.0)	36.0±7.8 (−9.0±9.0)	25.2±4.8 (−19.8±11.4)	p=0.936
	TAG	47.4±9.0 (0.0)	54.6±11.4 (7.2±6.0)	33.0±4.8 (−14.4±7.2)	31.2±9.6 (−16.2±7.8)	
Glucose (mmol/l)	DAG	8.3±0.9 (0.0)	7.8±0.8 (−0.4±0.3)*	6.8±0.8 (−1.5±0.3)*	6.1±0.6 (−2.2±0.4)	p=0.037
	TAG	8.8±0.8 (0.0)	7.5±0.7 (−1.3±0.2)	6.2±0.5 (−2.6±0.3)	5.9±0.4 (−2.9±0.6)	
Leptin (μg/l)	DAG	7.7±2.0 (0.0)	6.8±1.8 (−0.9±0.3)	6.4±1.8 (−1.4±0.3)	6.3±1.7 (−1.4±0.5)	p=0.741
	TAG	7.3±1.8 (0.0)	6.5±1.6 (−0.8±0.2)	5.9±1.4 (−1.4±0.4)	6.1±1.5 (−1.3±0.4)	
Preheparin LPL protein mass (g/l)	DAG	0.52±0.07 (0.00)	0.48±0.0 5 (−0.04±0.04)	0.45±0.05 (−0.07±0.04)	0.40±0.04 (−0.11±0.05)	p=0.460
	TAG	0.54±0.09 (0.00)	0.51±0.07 (−0.03±0.03)	0.49±0.07 (−0.06±0.03)	0.48±0.07 (−0.06±0.02)	
PAI-1 (μg/l)	DAG	17±3 (0)	16±3 (−1±4)	14±3 (−3±4)	9±1 (−8±3)	p=0.554
	TAG	15±3 (0)	17±3 (2±2)	14±2 (−1±2)	11±2 (−5±2)	
T-ketone bodies (μmol/l)	DAG	153±63 (0)	145±31 (−8±53)	443±74 (290±67)	506±108 (353±113)	p=0.074
	TAG	127±43 (0)	230±53 (102±69)	443±111 (315±118)	507±118 (380±112)	

Values are Mean±S.E. Mean±S.E. changes from baseline are shown in parentheses (Δ). P values are calculated by repeated-measures two-way ANOVA (Δ). DAG: diacylglycerol; TAG: triacylglycerol; LPL: lipoprotein lipase; T-ketone bodies: total ketone bodies.

* Significantly different from TAG ingesition at the same time points by paired t-test: $p<0.05$.

in comparison with those during DAG loading ($p<0.05$). The concentration of total ketone bodies in the serum was increased over time following either fat loading, but no significant difference was observed between the values for TAG and DAG loading. Plasma LPL protein mass, serum leptin, and plasma PAI-1 decreased over time after either fat loading, and there were no significant differences between these respective values in the TAG and DAG groups.

4. Discussion

The effects of DAG loading on postprandial changes in serum lipids and lipid parameters in diabetic patients were examined. Although the subject number of this study was small, the findings again showed the suppressed postprandial increases in serum TAG, RLP-TAG, and RLP-C in the DAG intake when compared with TAG intake in diabetic subjects as previously reported in healthy volunteers [15].

Increased fasting RLP-C concentrations have been reported in individuals with impaired glucose tolerance and in subjects with type 2 diabetes [1]. Ai et al. [28] reported that postprandial TAG and lipids in the RLP were also significantly increased in type 2 diabetics as compared with those in healthy subjects. In a recent report from the Framingham Study, RLP-C and RLP-TAG levels were shown to be higher in diabetic males and females, and these increases in RLP lipids were counted as a risk factor for CHD [29]. In the subanalysis of the Veterans Affairs HDL Intervention Trial (VA-HIT), a randomized controlled trial, Elam et al. [30] also reported that the incidence of CHD events was correlated positively to preprandial RLP-TAG and RLP-C levels. Furthermore, Mero et al. [31] suggested that postprandial changes in small remnant number might contribute to the severity of CAD in type 2 diabetes. These data suggest that the suppression of the postprandial increase in RLP lipids, achieved in our preliminary study by DAG loading, may be helpful to construct a nutritional therapeutic strategy, for reducing CHD risk, in diabetic patients.

The physicochemical mechanisms of the DAG oil-induced suppression of the postprandial increase in lipids have not yet been fully elucidated. During the processes of digestion, dietary TAG molecules are hydrolyzed to yield two free fatty acids and 2-monoacylglycerol (2-MAG) by intestinal lipases and absorbed into enterocytes. Triacylglycerol is then reassimilated again mainly via 2-MAG pathway in enterocytes [32]. On the other hand, 1,3-DAG, a major constituent of DAG, is hydrolyzed to glycerol and free fatty acids through 1-(or 3-)MAG during digestion. A portion of the 1-(or 3-)MAG enters enterocytes in the same manner as 2-MAG. However, resynthesis of TAG from 1-(or 3-)MAG will take

palace via the phosphatidic acid pathway, a slow turnover pathway, instead of the 2-MAG pathway. Hence, the lymphatic secretion of CM-TAG after the ingestion of DAG oil might be slower and possibly lower than that after the ingestion of naturally occurring TAG oil. These properties of 1,3-DAG may be beneficial in improving postprandial hyperlipidemia. Other metabolic characteristics of 1,3-DAG were discussed elsewhere recently [33].

As shown in Table 4, the increase in CM-TAG with DAG loading was slightly lower, and the time required for CM-TAG to reach the maximum level following DAG loading was longer as compared with TAG loading, although significant differences between the two groups were not detected. Eventually, however, these phenomena may lead to the significant suppression of the postprandial increase in serum TAG and RLP lipids after DAG loading.

Another aim of this experiment was to investigate the adverse effects of DAG intake especially on the formation of ketone bodies in diabetes. It is known that most diabetic patients have an abnormal ketone body metabolism. It was reported that higher activities of enzymes involved in the beta-oxidation pathway were detected with DAG feeding as compared with TAG feeding in the rat liver [34]. Increased fat oxidation was also detected in an experiment with 12 women who ingested DAG as compared with TAG oil [35]. Retarded TAG resynthesis in the enterocyte with DAG feeding, as described above, can result in an increased fatty acid concentration in enterocytes. Fatty acids unutilized for de novo TAG resynthesis in enterocytes may be utilized as energy. The retarded glucose reduction observed in our study during DAG loading as compared with TAG loading (Table 5) is conceivably due to the utilization of fatty acids instead of glucose as an energy source. This retarded glucose reduction during the DAG loading was also reported in healthy subjects by Taguchi et al. [36]. Thus, an increase in ketone bodies in the circulation following oral DAG loading in diabetics was our concern. However, this study showed no significant difference in the concentration of serum ketone bodies between the TAG and DAG groups for the moderately controlled diabetic patients with HbA1c levels <8%. Although we evaluated postprandial responses of single loading of DAG oil by the comparison with TAG oil using fat emulsion as a test food, oils in a typical diet should be evaluated as the next step with much larger population of diabetic subjects.

In summary, DAG ingestion in contrast to TAG ingestion suppressed postprandial increases in serum TAG, RLP-C, and RLP-TAG in six diabetics with moderate glycemic control. In addition, no significant differences were observed in postprandial changes in serum levels of insulin, free fatty acid, and ketone bodies between the DAG and TAG loading.

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Effects of bezafibrate on lipoprotein subclasses and inflammatory markers in patients with hypertriglyceridemia—a nuclear magnetic resonance study

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Abstract

Background: Hypertriglyceridemia is often associated with elevated remnants, small dense LDL and decreased HDL-cholesterol (C). The objective of this study was to investigate the efficacy of bezafibrate on lipoprotein subfractions profile and inflammation markers in patients with hypertriglyceridemia.
Methods: Twenty-four hypertriglyceridemic subjects took bezafibrate, 400 mg daily, for 4 weeks. Lipoprotein subclasses were measured by nuclear magnetic resonance (NMR) spectroscopy. Inflammation markers including C-reactive protein (CRP), interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) were also determined.
Results: Bezafibrate lowered triglyceride (TG) by 59% and increased HDL-C by 20%. NMR analysis revealed that bezafibrate lowered large TG-rich lipoproteins and IDL by 81% and 46%, respectively. Small LDL was selectively decreased by 53% with increase in large to intermediate LDL, thus altering the LDL distribution towards the larger particles (mean diameter 19.9 to 20.7 nm, p=0.0001). Small (HDL1) and intermediate (HDL3) HDL significantly increased by 168% and 70%, whereby resulting in a significant reduction of the mean HDL particle size from 9.0 to 8.7 nm (p=0.026). None of inflammation makers showed significant change by bezafibrate.
Conclusions: Bezafibrate effectively ameliorates atherogenic dyslipidemia by reducing remnants and small LDL as well as by increasing HDL particles in hypertriglyceridemic subjects.

Keywords: Bezafibrate; Lipoprotein; Nuclear magnetic resonance; Triglyceride; Inflammatory marker

1. Introduction

There is a growing body of evidence that, in addition to LDL-cholesterol (C), increased TG-rich lipoproteins (increased remnants), decreased HDL-C and small dense LDL comprise a new cluster of atherogenic dyslipidemia. This type of dyslipidemia represents metabolic perturbations as a consequence of abdominal obesity and involves metabolic abnormalities over entire lipoprotein spectrums and more importantly associates with the increased risk for coronary artery disease (CAD) [1].

Bezafibrate, one of fibrate derivatives, has been considered to be highly effective in treating hypertriglyceridemia and provided a benefit in CAD patients with high TG and low HDL-C levels [2]. Previous studies also revealed that bezafibrate increased HDL-C and altered LDL particles size in favor of decreasing small dense LDL [3,4]. While these studies all employed laborious ultracentrifugation and gradient gel electrophoresis methodologies to quantify lipoprotein subclasses, a new technology utilizing proton nuclear magnetic resonance (NMR) has drawn more attentions recently [5,6]. This new method uses signals emitted from methyl groups of lipids. Overall NMR signals from these lipids were computationally decomposed to yield 16 lipoproteins subclasses concentrations, together with particle numbers and sizes. The NMR method does not require fractionation of lipoproteins, is rapid measurement, and NMR-determined lipoprotein subclasses concentrations correspond well to

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those by the established methods [7]. Furthermore, recent studies [8–10] have shown clinical values of NMR-measured lipoprotein subclasses concentrations as the risk of CAD. In the present study, we, for the first time, utilized NMR method to investigate the effect of bezafibrate on lipoprotein metabolism in hypertriglyceridemic patients. We also focused on potential effects of bezafibrate on inflammatory markers.

2. Methods

Twenty four hypertriglyceridemic patients were recruited for this study from out-patients clinic of Jikei University School of Medicine Hospital. Eligibility criteria included age between 30 and 70 years, body mass index (BMI) less than 30 kg/m^2 and fasting triglyceride values $\geq$150 mg/dl both at screening and at the end of run-in period. None of the study subjects had poorly controlled diabetes (HbA1c>8%) or evidence of thyroid, liver or renal dysfunction (creatinine>2 mg/dl) and none were taking lipid-modifying medications. All subjects were instructed to maintain their diet, exercise and alcohol intake throughout the study period, including the run-in period. The study was approved by the Ethics Committee of Jikei University School of Medicine. All study subjects gave written informed consent to the study protocol.

After a 4–8-week run-in period, the patients received bezafibrate 200 mg twice daily for 4 weeks. Fasting blood samples (12-h overnight) were drawn from each subject at the end of run-in period and after 4-week bezafibrate treatment into tubes containing N_2EDTA at a final concentration of 0.1%. Plasma was immediately separated by centrifugation (3000 g, 20 min at 4 °C), then plasma concentrations of cholesterol and triglyceride (TG), and HDL-C [11] were freshly measured by the automated enzymatic technique using a Toshiba TBA-80FR auto-analyzer. LDL-C was directly measured using homogeneous enzymatic assay [12] from Daiichi Pure Chemicals (Tokyo, Japan). Plasma apolipoproteins concentrations were quantified using immunoturbidimetric assays [13]. Remnant-like particles (RLP)-C was measured by an immunoseparation technique (Japan Immunoresearch Laboratories, Takasaki, Japan) as reported previously [14]. Aliquots of plasma were stored at −80 °C for the following assays.

Malondialdehyde-modified LDL (MDA-LDL) was measured by ELISA using monoclonal antibody (ML25) as previously described [15]. Interleukin (IL)-6 and monocyte chemotactic protein-1 (MCP-1) levels were measured with an immunoassay using specific monoclonal antibodies (R&D Systems). Measurement of C-reactive protein (CRP) levels was obtained by a high sensitivity latex-enhanced immunonephelometics on the Behring BN II analyzer (Dade Behring) [16]. Frozen plasma was shipped to LipoScience (Raleiph, NC) for proton NMR analysis as previously reported [6,9]. This new method is based on the signals emitted from methyl group protons of the lipids, which were then calculated by linear least-square method to quantify lipoprotein subclasses lipids concentrations (TG for VLDL, C for IDL, LDL and HDL subclasses). Total of 16 subclasses were simultaneously measured: 6 VLDL subclasses (VLDL6 150±70 nm, VLDL5 70±10 nm, VLDL4 50±10 nm, VLDL3 38±3 nm, VLDL2 33±2 nm, VLDL1 29±2 nm), 4 LDL subclasses (IDL 25±2 nm, LDL3 22±0.7 nm, LDL2 20.5±0.7 nm, LDL1 19±0.7 nm) and 5 HDL subclasses (HDL5 11.5±1.5 nm, HDL4 9.4±0.6 nm, HDL3 8.5±0.3 nm, HDL2 8.0±0.2 nm, HDL1 7.5±0.2 nm). LDL and HDL particle concentrations (nanomoles per liter) are calculated as the sum of LDL subclasses concentrations including IDL for the former and the sum of HDL subclasses for the latter. Average lipoprotein particle sizes were determined by the weighting the mass percentage of each subclass by its diameter. LDL sizes by NMR method closely correlate with those by gradient gel electrophoresis (GGE) method, but are smaller by 5 nm. This resulted in 20.5 nm as a good cutoff point to distinguish individuals with pattern A (mainly large LDL) and pattern B (mainly small LDL), giving a good agreement to the phenotyping by original GGE method.

ApoE phenotype was determined by immunoblot using a specific goat anti-apoE polyclonal antibody as reported previously [17].

Among lipid parameters, TG showed a skewed distribution. Therefore, logarithmically transformed TG values were used for comparison. Changes in biochemical parameters with bezafibrate treatment were analyzed by the paired *t*-test. Distribution of LDL size (patterns A and B) was tested by a chi-square test. Pearson's correlation coefficients were calculated to examine the relationship between lipid parameters. A *p*-value of less than 0.05 with a two-tailed test was considered to be statistically significant. All statistical procedures were performed using SPSS software (version 9.1, SPSS Chicago IL).

3. Results

3.1. Baseline demographic measures

Baseline characteristics of the study subjects are summarized in Table 1. The study subjects were mostly comprised of men (91.7%) and the average age of 53.8 years old. The average BMI of 25.1 kg/m^2 was equal to the cut-off point to define obesity in Japan and, in fact, 10 patients (42%) were obese. Percentage of hypertension and diabetes are 50% and 25%, respectively. Current smokers comprised 42%. All patients, except for one patient who underwent coronary artery bypass surgery, were free from coronary artery disease evidenced by clinical symptoms, electrocardiogram or laboratory data abnormalities.

3.2. Changes in lipid parameters

Plasma lipids and lipoproteins concentration at baseline and bezafibrate treatment are also listed in Table 1. Descriptive values of total cholesterol ranged from 173 to 391 mg/dl with the mean of 239 mg/dl. Likewise, TG ranged from 150 to 1553 mg/dl with the mean of 427 mg/dl, HDL-C from 25 to 73 mg/dl with the mean of 40.6 mg/dl and LDL-C from 69 to 265 mg/dl with the mean of 124 mg/dl, respectively, thus representing type IV hyperlipoproteinemia as the primary lipid abnormality in the present cohort.

Logarithmically transformed TG negatively correlated with HDL-C, but not LDL-C levels. Bezafibrate significantly reduced total cholesterol, TG and significantly increased HDL-C. In parallel to the changes in lipids, apoC-II, apoC-III and apoE all significantly decreased, whereas apoA-I and apoA-II increased significantly. It should be noted that the magnitudes of change of apoC-III and apoA-II were greater than those of other apolipoproteins.

3.3. Lipoprotein subclasses analysis by NMR

Effects of bezafibrate on lipoprotein subclasses parameters by NMR are summarized in Table 2. Bezafibrate significantly reduced VLDL particles, in particular, large VLDL (VLDL4 to 6) with little effects on relatively small VLDL (VLDL1 to 3). These resulted in a significant reduction of mean VLDL particle size. Although not significant, bezafibrate reduced IDL by 46.4%. Bezafibrate also produced significant increases in large (LDL3) and intermediate (LDL2) LDL, contrasting an effect on small LDL (LDL1) which showed a significant 52.5% decrease. This shift of LDL subclasses distribution was translated into a significant increase in particle size as well as a significant decrease of LDL phenotype pattern B. Therefore, 11.7% increase in LDL-C was a product of 3.9% enlargement of particle size and 4.6% reduction of particle number. Bezafibrate caused a selective and significant increase in the levels of intermediate (HDL3) and small (HDL1) HDL. Whereas HDL2, HDL4 and HDL5 did not change significantly by bezafibrate, thus resulting a significant decrease in average HDL particles size and a significant increase in HDL particle number.

Table 1
Characteristics of study subjects and changes in lipids with bezafibrate

	Baseline	Bezafibrate	Change (%)
N	24		
Age (years)	53.8±12.7		
Sex (% men)	92%		
BMI (kg/m^2)	25.1±3.1		
Current smoker (%)	42%		
Hypertension (%)	50%		
Diabetes (%)	25%		
TC (mg/dl)	239±51	221±47	−7.7†
TG (mg/dl)	427±321	176±97	−58.8‡
HDL-C (mg/dl)	40.6±11.5	48.6±12.5	19.8‡
LDL-C (mg/dl)	124±44	139±38	11.4
ApoA-I (mg/dl)	126±24	135±21	6.8*
ApoA-II (mg/dl)	27.1±5.3	36.5±7.6	34.8‡
ApoB (mg/dl)	117±25	112±34	−4.0
ApoC-II (mg/dl)	9.9±8.4	6.6±3.4	−33.9*
ApoC-III (mg/dl)	22.9±15.4	12.3±5.9	−46.4†
ApoE (mg/dl)	8.5±6.2	5.4±1.8	−36.5†
RLP-C (mg/dl)	25.5±22.8	9.3±6.0	−63.5*

Values are given as the mean±S.D., except values in percentage.
* $p<0.05$.
† $p<0.01$.
‡ $p<0.001$.

Table 2
Effects of bezafibrate on lipoprotein subclasses parameters by NMR

	Baseline	Bezafibrate	Change (%)
VLDL (mg/dl triglyceride)			
VLDL6 (large)	38.2±60.8	0.7±1.6	−98.2†
VLDL5	128.0±204.2	30.3±49.7	−76.4*
VLDL4	125.4±122.9	63.8±53.4	−49.1†
VLDL3	28.0±40.3	20.1±31.8	−28.3
VLDL2	8.0±9.8	8.5±8.4	7.0
VLDL1 (small)	5.1±7.0	5.6±6.8	10.0
VLDL particle size (nm)	53.6±13.1	44.9±6.9	−16.4†
LDL (mg/dl cholesterol)			
IDL	8.7±9.9	4.6±6.3	−46.4
LDL3 (large)	30.4±48.1	58.0±39.5	90.6*
LDL2	26.4±33.4	47.1±36.9	78.3*
LDL1 (small)	68.2±53.0	32.3±50.5	−52.5*
LDL particle size (nm)	19.9±1.0	20.7±0.7	3.9‡
LDL particle number (nmol/l)	1722±629	1643±637	−4.6
LDL phenotype[a]			
Pattern A (*n*)	5	16	p=0.0014
Pattern B (*n*)	19	8	
HDL (mg/dl cholesterol)			
HDL5 (large)	8.3±6.4	7.3±5.5	−11.6
HDL4	10.1±5.9	11.4±5.1	12.9
HDL3	5.8±5.3	9.8±7.4	70.1*
HDL2	15.0±6.0	16.3±6.3	8.3
HDL1 (small)	1.4±3.6	3.7±5.4	167.9*
HDL particle size (nm)	9.0±0.5	8.7±0.4	−3.2*
HDL particle number (μmol/l)	26.9±7.2	34.7±7.4	28.8‡

[a] The average LDL particle size of 20.5 nm by NMR was used as the cutoff point to distinguish individuals with pattern A (mainly large LDL) and pattern B (mainly small LDL).
Values are given as mean±S.D.
* $p<0.05$.
† $p<0.01$.
‡ $p<0.001$.

3.4. Correlation study using lipoprotein concentrations and particle sizes and their changes after bezafibrate treatment

Fig. 1 illustrates correlation between TG, HDL concentrations and particle sizes. Plasma TG concentration correlated negatively with LDL size, but not with HDL size determined by NMR. HDL-C level showed a signifi-

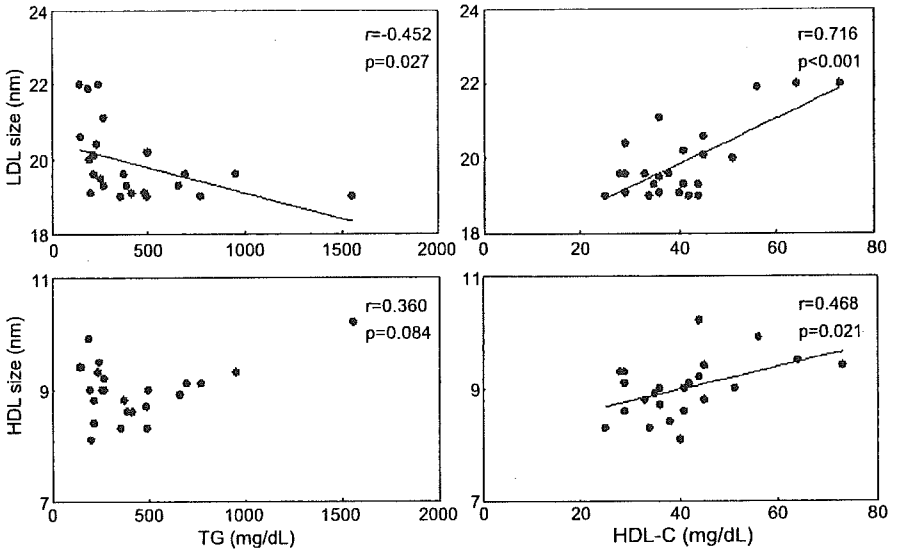

Fig. 1. Correlation between lipid parameters (TG and HDL-C) and particles sizes (LDL and HDL) in study subjects.

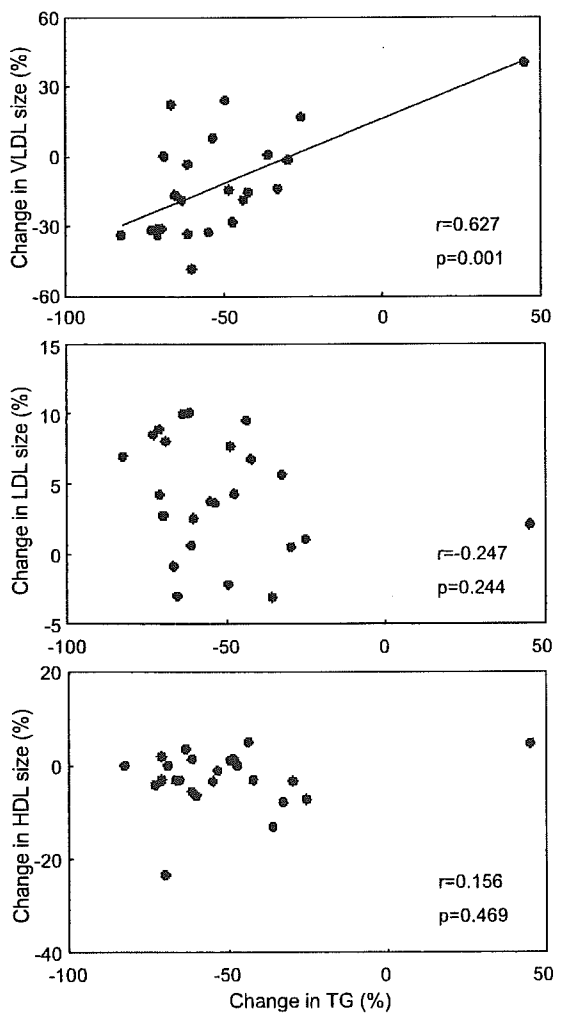

Fig. 2. Correlation between changes in TG and changes in particles sizes (VLDL, IDL and HDL) after bezafibrate treatment.

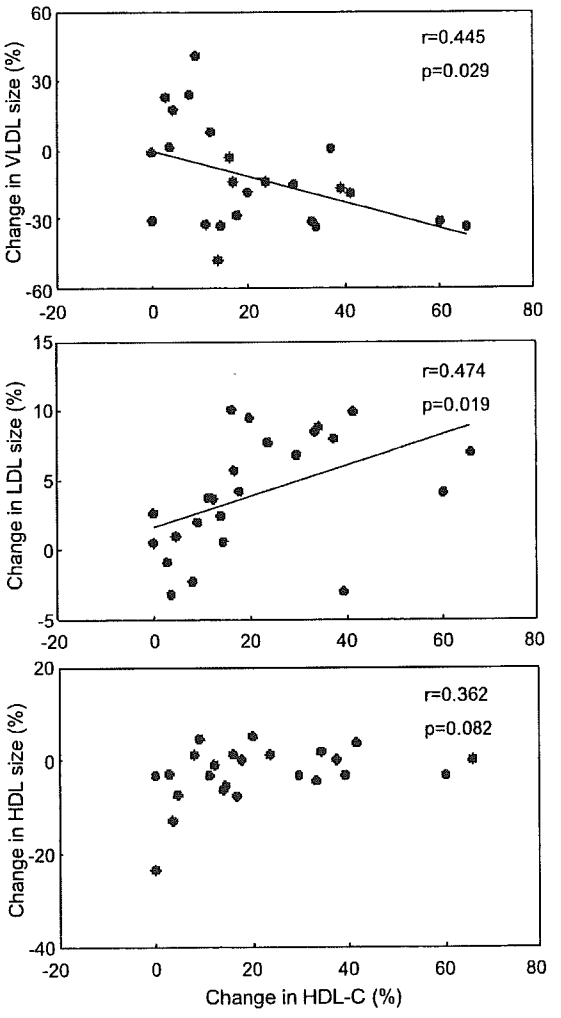

Fig. 3. Correlation between changes in HDL-C and changes in particles sizes (VLDL, IDL and HDL) after bezafibrate treatment.

Table 3
Effects of bezafibrate on MDA-LDL concentration and inflammation markers

	Baseline	Bezafibrate	Change (%)
MDA-LDL (U/l)	91.7±25.8	88.8±33.7	−3.2
MDA-LDL/apoB ($U/mg/10^{-1}$)	0.796±0.151	0.784±0.163	−1.6
hsCRP (mg/l)	1.13±1.50	1.02±1.44	−10.1
PAI-1 (ng/ml)	73.1±64.1	80.8±29.9	10.6
IL-6 (pg/ml)	1.9±1.1	1.8±1.3	−4.5
MCP-1 (pg/ml)	132.2±40.7	121.3±44.5	−8.2

Values are given as mean±S.D.

cant and positive correlations with both LDL and HDL particles size. To further understand the relation between changes in TG, HDL-C and changes in lipoprotein particle size, we correlated percentage changes in lipid parameters and those in particles sizes as shown in Fig. 2 and 3. Changes in TG levels, mostly reduction except one patient, were shown to be correlated with changes in VLDL size, but not with changes in LDL size, indicating that enlargement of LDL particle size was independent of TG-lowering effect by bezafibrate. This relation was not affected by the outliner. Changes in HDL-C showed a strong relation with changes in particles sizes as shown in Fig. 3. The changes in HDL-C negatively correlated with those in VLDL particle size, but positively with those in LDL particle size, while marginally failed to show a significant correlation with those in HDL size.

3.5. *Inflammation markers*

Finally, effects of bezafibrate on inflammation markers are summarized in Table 3. Although small LDL (LDL1) significantly decreased by NMR analysis, neither MDA-LDL or MDA-LDL/apoB did not change by bezafibrate treatment. Likewise, high sensitivity CRP, PAI-1, IL-6 or MCP-1did not show significant changes by bezafibrate treatment.

4. Discussion

In addition to elevated LDL-C, increased TG-rich lipoproteins (increased remnants), decreased HDL-C and small dense LDL comprise a new cluster of atherogenic dyslipidemia. Unlike hypercholesterolemia in which HMG-CoA reductase inhibitors are now widely accepted as the first choice, fibrate derivatives are considered to be an ideal treatment for this type of dyslipidemia. A recent intervention trials further supported this strategy by demonstrating that gemfibrozil provided a benefit when used in CAD patients with low HDL-C levels [18]. In the present study, we employed, for first time in Japan, proton nuclear magnetic resonance spectroscopy for lipoprotein subclasses analysis and herein demonstrated that bezafibrate markedly reduced TG-rich lipoproteins, including atherogenic IDL, increased small HDL particle number and altered LDL particles size in favor of converting small LDL dominant pattern B to large LDL dominant pattern A.

Besides elevated LDL-C, small dense LDL has been a new member as independent risk factor for CAD [19]. LDL phenotype pattern B was dominant (79.2%) prior to the bezafbrate treatment, but markedly decreased to 33.3% by the end of 4 weeks treatment, mirroring a significant decrease in small LDL (LDL1) as well as increases in intermediate (LDL2) and large LDL (LDL3). This is consistent to the previous findings in which LDL particle size was evaluated by GGE method [3, 4]. Enlargement of LDL is supposedly translated to be less atherogenic, whereby counterbalancing the mild elevation of LDL-C by bezafibrate.

Although baseline TG concentrations, together with HDL-C levels, are determinants for LDL particle sizes (Fig. 1), changes in LDL particle size after bezafibrate treatment was not correlated with those in TG level (Fig. 2), indicating that enlargement of LDL by bezafibrate is not primarily mediated by TG lowering function. Interestingly, enlargement of LDL did correlate with the increase in HDL-C level. This is a puzzling observation based on a significant inverse correlation between changes in TG and HDL-C ($r=-0.47$, $p=0.02$) by bezafibrate, which indicates that TG-rich lipoproteins and HDL may be metabolically associated. In contrast to our correlation results, Hirano et al. [4] found that an increase in LDL size was associated with a decrease in TG level, but not with an increase in HDL-C level. At present, we are not certain about the exact reasons underlying this discrepancy. Since sample sizes in both studies are relatively small (24 in this study and 17 in their study), this could be due simply to "by chance". There are, however, some differences in baseline lipid profiles and responses to bezafibrate treatment by which the discrepancy might be explained. Our study subjects had higher TG levels (427 vs. 195 mg/dl as the mean) and lower LDL-C levels (124 vs. 148 mg/dl) as compared with their study subjects. LDL-C modestly increased in the present study (+11.7%), whereas decreased in their study (−13%). Despite similar baseline HDL-C levels, HDL-C increased to a greater extent (+44%) in their study relative to the present study (+19.8%). A closer look on TG response to bezafibrate (Fig. 3 in their manuscript) also indicates that their study subjects show larger variations relative to the present study subjects, thus leading to a greater probability of changes in TG to significantly associate with changes in LDL size. Conversely, as evident from Figs. 2 and 3, our study subjects showed greater variation in HDL-C response to bezafibrate relative to TG. In our opinion, there could be several potential explanations for the positive relation between LDL size enlargement and HDL elevation by bezafibrate. First, if HDL-C elevation is due to the suppression of cholesteryl ester transfer protein activity as reported previously [20], this, in turn, affects lipids exchange among VLDL, LDL and HDL, whereby resulting to modulate LDL particle size.

Second, bezafibrate affects lipoprotein lipase including lipoprotein lipase and hepatic triglyceride lipase, lipases known to be involved in synthesis and remodeling of both LDL and HDL. These mechanisms remain to be speculations since we did not measure these enzyme activities or masses, thus deserving a future study.

In the present study, we confirmed a favorable and potent effect of bezafibrate on HDL metabolism as evidenced by the 19.8% increase in HDL-C. NMR analysis further extended this effect by demonstrating that bezafibrate markedly increased intermediate and small HDL subclasses with the average HDL size to be significantly decreased from 9.0 to 8.7nm. This finding is compared favorably to the increase in HDL_3 evaluated by GGE methods in previous studies [3,20]. An increase in preβ1-HDL at the expense of HDL_{2b} using native two-dimensional gel electrophoresis [21] also supports our finding. These small HDL are believed to be better acceptors for cholesterol as compared with larger counterparts, as evidenced by a finding that free cholesterol effluxing capacity was markedly increased by bezafibrate treatment [22]. Overall, the decrease in HDL size, together with the increase in HDL particle number, makes bezafibrate to be an ideal agent to strengthen anti-atherogenic properties of HDL when used in hypertriglyceridemic patients.

Of note is a marked reduction of RLP-C, a parameter of atherogenic remnants, in this study. Although not significant, this favorable effect is further supported by NMR analysis showing 46% reduction. Since development of CAD is not always associated with elevated LDL-C [23], improved remnant metabolism, together with favorable effect on HDL metabolism, may account for the benefit observed in the recent intervention trials using fibrate [2,18].

Lundman et al. [24] reported an increased plasma concentration of CRP in hyperlipidemic subjects. This is in agreement to our study in which the average CRP level (1.13 mg/l) are comparable to that study (1.5 mg/l). Bezafibrate, however, did not modulate CRP concentration in this study. The effect of bezafibrate on CRP is controversial with positive [25] and negative [26] results. In previous studies [25,27], bezafibrate was found to reduce IL-6, an effect being attributable to the activation of the peroxisome proliferator-activated receptor (PPAR)-α with a consequent reduction of NF-κβ activation. The IL-6 levels, however, remained unchanged in this study. These discrepancies may be due to the differences in inflammatory status at baseline, duration of the study period, or the lack of control group. The exact reasons for these observations are not clear at present, thus deserving a placebo-control and long term follow-up study in future.

There are some limitations in the present study. First, this study lacks control group to which bezafibrate group can be compared. We therefore installed run-in period to maintain steady state throughout the study period. Nonetheless, placebo-control study should be carried out to draw a solid conclusion in future. Second, most of the study subjects were male, thus it should be cautious to extend our results to hypertriglyceridemic women. Third, duration of the treatment was relatively short (4 weeks). In this regard, our results are considered to be short-term effects, which may be different from long-term effects.

In summary, the results of the present study demonstrated that, in addition to its potent TG lowering effect, bezafibrate effectively improved proatherogenic lipoproteins profiles by reducing remnants, small LDL as well as increasing small HDL particles. Therefore, it will be of great importance as to whether bezafibrate may provide a long-term benefit in hypertriglyceridemic patients at risk for coronary artery disease, typically patients with metabolic syndrome.

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Roles of degree of fat deposition and its localization on VEGF expression in adipocytes

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Miyazawa-Hoshimoto, Saori, Kazuo Takahashi, Hideaki Bujo, Naotake Hashimoto, Kazuo Yagui, and Yasushi Saito. Roles of degree of fat deposition and its localization on VEGF expression in adipocytes. *Am J Physiol Endocrinol Metab* 288: E1128–E1136, 2005. First published December 21, 2004; doi:10.1152/ajpendo.00003.2004.—Vascular endothelial growth factor (VEGF) is an important angiogenic factor and is expressed in wide variety of cell types. In this study, we investigated the mechanism of VEGF production in adipocytes in three sets of experiments. First, to clarify the relation between plasma VEGF concentrations and their expressions in adipose tissues, we investigated the genetically obese *db/db* and KK-A^y mice. Plasma VEGF concentrations in obese mice were significantly higher than in control and were related to adiposity. VEGF expressions in visceral fat were enhanced during growth and were related to fat deposition. Next, to demonstrate the relation between VEGF production and lipid accumulation in adipocytes, we analyzed VEGF mRNA expression and its protein secretion in 3T3-L1 cells. VEGF production was enhanced during lipid accumulation in 3T3-L1 cells after adipocyte conversion. Next, to clarify the role of anatomic localization on VEGF expression in adipocytes, we implanted 3T3-L1 cells into visceral or subcutaneous fat in athymic mice. 3T3-L1 cells implanted into the mesenteric area expressed more VEGF mRNA than that into the subcutaneous area. Plasma VEGF concentration in the mice implanted in visceral fat was higher than in controls. These results suggest that both the anatomic localization and the lipid accumulation are important for the VEGF production in adipocytes.

vascular endothelial growth factor; fat distribution; cytokine; gene expression; 3T3-L1 cells

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) is a very potent angiogenic factor that induces migration and proliferation of vascular endothelial cells (9). VEGF also enhances vascular permeability and modulates thrombogenicity (18). It has therefore been implicated in normal blood vessel development as well as in pathological vessel formation (6). Pathogenic neovascularization plays a major role in the development of atherosclerosis (20), tumor growth (18), rheumatoid arthritis (9, 16), and various retinopathies (2, 3). VEGF mRNA expression has been identified in various cell types, including endothelial, epithelial, and mesenchymal cells (9, 18). It has also been reported that VEGF mRNA is expressed in 3T3-F442A cells, an established preadipocyte cell line (4).

VEGF is encoded by a single gene; however, four isoforms of 205, 188, 164, and 120 amino acids long are produced as a result of alternative splicing. The 164-amino acid-long isoform is the most abundant. We have reported that serum concentration of the 164-amino acid-long isoform of VEGF in human obese subjects is dependent on the intra-abdominal fat accumulation determined using computed tomography scan at the umbilical level (15). Furthermore, the elevated VEGF level or the accumulated visceral fat in the obese subjects was decreased after body weight reduction (15). These observations revealed that the VEGF secretion from adipose tissues, particularly from visceral adipose tissues, might regulate its serum concentration.

Adipose tissues have been reported to express and release various secretary molecules, such as leptin (10, 11), tumor necrosis factor-α (TNF-α) (7), plasminogen activator inhibitor-1 (PAI-1) (20), and IL-6 (22). Especially, the expression levels of TNF-α and PAI-1 in adipocytes are shown to be directly related to the degree of differentiation from preadipocytes and to be dependent on their anatomic location (12, 14, 20, 21).

Therefore, it is very important to clarify the mechanism of VEGF production in adipocytes from the point of view of the adipocyte differentiation process and the site of fat accumulation. In this study, we examined VEGF production from intrinsic adipocytes in *db/db* and KK-A^y mice during growth, from 3T3-L1 cells depending on the differentiation in culture, and from 3T3-L1 cells that were implanted into the visceral or subcutaneous fat area.

MATERIALS AND METHODS

Materials. MCDB131, FBS, and trypsin were purchased from Invitrogen (Carlsbad, CA). PBS was from Nissui Pharmaceuticals (Tokyo, Japan). DMEM, human insulin, dexamethasone, and 3-isobutyl-1-methylxanthine were obtained from Sigma-Aldrich (St. Louis, MO). Collagenase-S1 was from Nitta Gelatin (Osaka, Japan). Growth Factor Reduced BD Matrigel matrix was from Nippon Becton-Dickinson (Tokyo, Japan). ISOGEN reagent was from NIPPON GENE (Tokyo, Japan). RNeasy Mini Kits and QIAGEN OneStep RT-PCR Kit were from Qiagen (Tokyo, Japan). 3T3-L1 cells, an established preadipocyte cell line, was obtained from the American Type Culture Collection (Manassas, VA). Human umbilical vein endothelial cells (HUVECs) and EBM-2 medium were from BioWhittaker (Walkersville, MD).

Obese mice. BKS.Cg-+ Lepr^{db}/+ Lepr^{db}/Jcl (*db/db*) and KK-A^y/TaJcl (KK-A^y) mice, and control littermates BKS.Cg-m +/+ Lepr^{db}/Jcl (*db/+*) and C57BL/6JJcl (C57BL/6), respectively, were obtained from CLEA Japan (Tokyo, Japan) at 5 wk of age. The mice were maintained in a temperature-, humidity-, and light-controlled room (12:12-h light-dark cycle) with free access to water and standard

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