Table 3. Comparisons of Clinical Data During the Follow-Up Period Between Patients With and Without ISR

	With ISR (n = 46)	Without ISR (n = 95)	p Value
Risk status at the follow-up			
Total cholesterol (mg/dl)	186 ± 33	183 ± 32	NS
LDL cholesterol (mg/dl)	109 ± 28	104 ± 28	NS
HDL cholesterol (mg/dl)	54 ± 13	53 ± 12	NS
HbA1c (mg/dl)	5.9 ± 1.1	5.8 ± 1.1	NS
hsCRP (mg/dl)	0.17 ± 0.16	0.15 ± 0.17	NS
Systolic blood pressure (mm Hg)	121 ± 15	126 ± 20	NS
BMI (kg/m^2)	24 ± 2.6	24 ± 2.9	NS
Change of risk status from baseline			
Change of total cholesterol (%)	−4.7 ± 19	−6.2 ± 17	NS
Change of LDL-C (%)	−5.9 ± 29	−9.7 ± 26	NS
Change of HDL-C (%)	8.7 ± 2.2	7.3 ± 1.8	NS
Change of HbA1c (%)	−3.2 ± 1.1	−2.3 ± 1.2	NS
Change of hsCRP (%)	−26 ± 32	−32 ± 60	NS
Change of SBP (%)	−5.1 ± 1.8	−5.1 ± 1.7	NS
Change of BMI (%)	−6.1 ± 1.9	−5.3 ± 2.5	NS
Cessation of smoking (%)	61 (11/18)	66 (21/32)	NS
Increase in physical activity (%)	18	20	NS
Chest pain			
Asymptomatic (%)	54	87	<0.01
CCS class			
I (%)	22	11	<0.01
II (%)	7	2	<0.05
III (%)	4	0	
IV (%)	3	0	

Data are expressed as mean ± SD or percentage of the patients.
BMI = body mass index; CCS = Canadian Cardiovascular Society angina classification class (10); HbA1c = glycosylated hemoglobin; SBP = systolic blood pressure. Other abbreviations as in Tables 1 and 2.

and without ISR, and the initial FMD was lower in patients with or without ISR than in the age- and gender-matched control subjects (Fig. 1). In patients without ISR, the FMD at the end of the six-month follow-up period improved to levels comparable to the control subjects, whereas the follow-up FMD in patients with ISR did not significantly change compared with the initial FMD (Fig. 1). As a result, the follow-up FMD was lower in patients with ISR than without ISR. Dilator responses to nitroglycerin, brachial

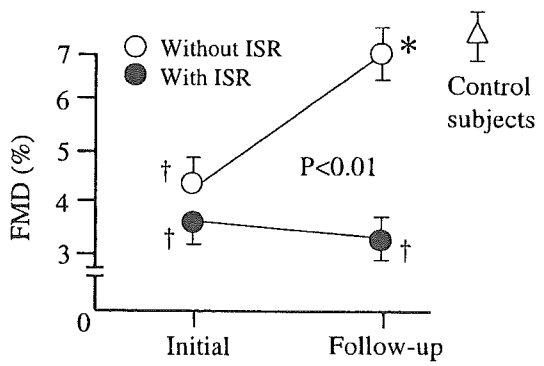

Figure 1. Comparison of flow-mediated dilation (FMD) of the brachial artery at coronary stenting (Initial) and at the six-month follow-up (Follow-up) between patients with (n = 46) and without (n = 95) in-stent restenosis (ISR). The control subjects (n = 48) with normal coronary angiograms were selected to match age and gender of the patients with stenting and served as comparison of FMD with the patients. Data are expressed as mean ± SE. *p < 0.01 versus initial FMD in patients without ISR; †p < 0.01 versus control subjects.

arterial diameter at baseline, and brachial blood flow at baseline, and increase in the blood flow at reactive hyperemia were not significantly different among control subjects and patients with or without ISR at either the initial time or the end of the follow-up period (Table 4). The extent of the improvement of the follow-up FMD from the initial FMD in patients without ISR significantly correlated with the percentage changes in levels of total cholesterol ($r = -0.36$, $p < 0.01$), HDL-C ($r = 0.34$, $p < 0.05$), HbA1c ($r = -0.37$, $p < 0.05$), and hsCRP ($r = -0.32$, $p < 0.05$) from baseline to follow-up, whereas there was no significant correlation in patients with ISR (data not shown).

Association of the angiographic findings and FMD. The late luminal loss significantly and inversely correlated with the follow-up FMD, whereas it did not correlate with the initial FMD (Fig. 2). Furthermore, the late luminal loss inversely correlated with changes in the follow-up FMD from the initial FMD (Fig. 2). In multivariate logistic statistical analysis, ISR at the six-month follow-up period was most strongly associated with the impairment of the follow-up FMD (Table 5). This association was independent of the infarct-related artery, longer lesion length, multiple lesions with stenting, longer stented segment length, chest pain during the follow-up period, and a positive exercise stress test at the end of the six-month follow-up period (Table 5). Also, TLR was significantly associated with the impairment of the follow-up FMD

Table 6. Sensitivity and Specificity for Identification of ISR

			Impairment of Follow-Up FMD		
	Chest Pain (n = 141)	Positive Exercise Test (n = 116)	All Patients (n = 141)	Patients With Chest Pain (n = 33)	Patients Without Chest Pain (n = 108)
Sensitivity (%)	46 (21/46)	54 (22/41)	69* (32/46)	90*† (19/21)	52 (13/25)
Specificity (%)	87 (83/95)	77 (58/75)	75 (71/95)	83 (10/12)	73 (61/83)
Positive predictive value (%)	63 (21/33)	56 (22/39)	57 (32/56)	90*†‡ (19/21)	37 (13/35)
Negative predictive value (%)	77 (83/108)	75 (58/77)	84 (71/85)	83 (10/12)	84 (61/73)
Accuracy (%)	74 (104/141)	69 (80/116)	73 (103/141)	87 (29/33)	67 (74/108)

*$p < 0.05$ versus chest pain; †$p < 0.05$ versus positive exercise test; ‡$p < 0.05$ versus impairment of follow-up FMD in all patients.
ISR = in-stent restenosis; FMD = flow-mediated dilation.

The favorable changes in some of these risk factors, such as total cholesterol levels, HDL-C levels, HbA1c, and hsCRP, were significantly related to the improvement of FMD at follow-up in patients without ISR. These treatment-related reductions in the atherogenic burden improved endothelial vasomotor dysfunction, leading to attenuation of the intimal thickening of coronary arteries after stenting in patients without ISR; the reduction in these risk factors, however, failed to improve FMD in patients with ISR in spite of the similar treatments and reductions in risk factors. The reversibility of endothelial dysfunction in response to reduction in atherogenic burden might vary among individuals (17). The mechanisms that might explain the different responses of the follow-up FMD to the similar anti-atherosclerotic treatments and atherosclerotic burden between patients with and without ISR remain unknown. Unknown or other risk factors, not examined in the present study, might importantly affect reversibility of endothelial dysfunction in response to reduction in atherosclerotic burden. The normalization of endothelial dysfunction could be related to suppression of the intimal growth within stent after PCI, leading to attenuation of ISR; however, persistent impairment of endothelial dysfunction might fail to suppress it.

Although risk factor status at the end of the six-month follow-up period in patients without ISR was similar or still somewhat high compared with control subjects, the follow-up FMD in patients without ISR was improved to levels comparable to the control subjects. This might be explained by the higher frequencies in use of statins, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers, which have pleiotropic endothelial protective actions, in these patients than in control subjects (data not shown).

The present study also showed that the sensitivity of FMD impairment at follow-up in detecting late ISR was significantly higher or nearly higher than chest pain during the follow-up period and the positive exercise ECG test at the end of the follow-up period. The specificity among these three assessments was comparable. Furthermore, FMD impairment at follow-up in combination with chest pain during follow-up resulted in an incremental improvement in sensitivity (90% in patients with impaired FMD and chest-pain). Although the combined assessment did not significantly improve the specificity (83%) compared with each individual assessment, the sensitivity and specificity of the presence of impaired FMD at follow-up in combination with chest pain during follow-up appeared similar to the 70% to 95% sensitivity and 70% to 95% specificity that has been reported for detection of late ISR with stress myocardial perfusion scintigraphy (1,18). Also, results that are similar to ISR were obtained with TLR. The assessment of FMD is easily applied in clinical practice and requires only conventional equipment for vascular ultrasonography. Thus, the assessment of endothelial vasomotor dysfunction of the brachial artery during the follow-up period was useful for the identification of late ISR and TLR after stenting, especially when combined with the assessment of chest pain or the stress tests.

A previous report (19) showed an association between ISR and vasodilator dysfunction of forearm resistance vessels in a smaller number of patients. Unfortunately, this report failed to evaluate the influence of clinical, angio-

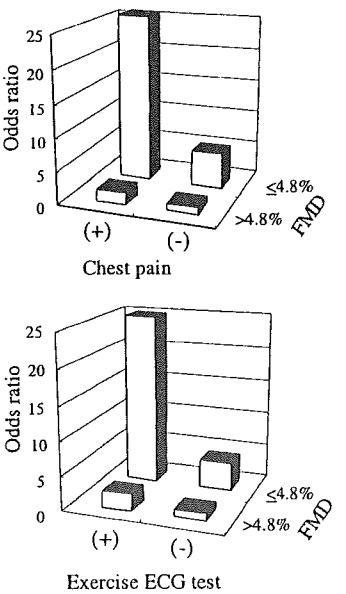

Figure 3. Incremental effects on the relative risk for late in-stent restenosis of the combination of impairment of follow-up flow-mediated dilation (FMD) with chest-pain positive **(upper panel)** and positive exercise electrocardiographic test **(lower panel)**.

graphic, and procedural factors on the association between ISR and vasodilator function with multivariate statistical analysis. In addition, there was no information reported with regard to the clinical usefulness of the association between ISR and vasodilator function. In another recent report (20) published during preparation of our present paper, an impairment of FMD at 30 days after PCI predicted ISR in patients with recurrent anginal symptoms or inducible myocardial ischemia at the six-month follow-up, although our study showed that an impairment of FMD before PCI did not predict ISR. It is possible that local coronary injury with PCI might affect their positive association of impaired FMD at 30 days after the procedure with ISR (21). Furthermore, this report (20) repeated the follow-up angiography only in patients with inducible ischemia (17% of the total patients), thereby the association between an impaired FMD and the angiographic ISR was not precisely determined in the study (20).

Although drug-eluting stents limit ISR, it remains to be determined whether endothelial dysfunction in systemic arteries might be also associated with ISR with drug-eluting stents, especially in high-risk patients with complex lesions.

In conclusion, the impairment of FMD at the follow-up time was independently and closely associated with late ISR after stenting in the native coronary arteries. The assessment of FMD at the time of follow-up might be useful for identification of late ISR after stenting.

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Remnant lipoproteinemia is a risk factor for endothelial vasomotor dysfunction and coronary artery disease in metabolic syndrome

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Abstract

This study aimed to determine whether elevated levels of remnant lipoprotein, an atherogenic triglyceride-rich lipoprotein, might be associated with coronary artery disease (CAD) and endothelial vasomotor dysfunction in metabolic syndrome. The fasting serum levels of remnant lipoproteins (remnant-like lipoprotein particles cholesterol; RLP-C) were measured by an immunoseparation method in 210 patients with metabolic syndrome meeting ATP III criteria. Flow-mediated endothelium-dependent dilatation (FMD) in the brachial artery during reactive hyperemia was examined by high-resolution ultrasound technique. This study found that elevated RLP-C levels were a significant and independent risk factor for impaired FMD and angiographically proven coronary artery disease (CAD). Treatment with bezafibrate ($n=20$) or atorvastatin ($n=20$) for 4 weeks significantly reduced RLP-C levels, with a concomitant improvement in FMD. The % reduction in RLP-C levels from baseline after the treatment was independently correlated with the magnitude of improvement in FMD after adjustment for the % changes in levels of triglyceride, hsCRP, and IL-6, and HOMA index. Thus, elevated levels of RLP-C are a risk factor for CAD and endothelial vasomotor dysfunction, a predictor of coronary events, in metabolic syndrome. Measurement of RLP-C is useful for assessment of CAD risk and therapeutic effects in metabolic syndrome.

Keywords: Remnant lipoproteins; Coronary artery disease; Endothelium-dependent vasodilation; Atherosclerosis; Metabolic syndrome

1. Introduction

Metabolic syndrome is a clustering of atherosclerotic metabolic abnormalities characterized by insulin resistance, visceral adiposity, high triglyceride, and low high-density lipoprotein (HDL). This syndrome is highly prevalent (40% of the population in USA >60 years of age) [1,2] and strongly associated with cardiovascular diseases (CVD) [3,4]. Therefore, it is important to target prevention strategies for patients with metabolic syndrome. However, multiple metabolic disorders contribute to the pathogenesis of this syndrome [1,5]. Furthermore, these metabolic disorders are intimately linked with each other, and thus the primary pathogenesis of this syndrome is difficult to determine in each patient. It also remains to be determined which metabolic disorders should be primary the therapeutic targets to prevent CVD and which metabolic disorders should be monitored to follow therapeutic effects.

Dyslipidemia, characterized by elevated triglycerides levels and low HDL levels, is a hallmark of metabolic syndrome [6,7]. We have shown that remnant lipoproteins, triglyceride-rich lipoproteins, are a strong risk factor for CVD [8–13]. Recently, a simple and reliable technique for measurement of remnant-like lipoprotein particles cholesterol (RLP-C) using an immunoseparation method has been developed [8,14]. We

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and others have shown that high RLP-C levels are a strong risk factor for CVD [8–11]. We thus hypothesized that measurement of RLP-C levels might be helpful in the assessment of CVD risk in metabolic syndrome. A number of studies have shown that endothelial vasomotor dysfunction, a predictor of future coronary events, is often present in patients with metabolic syndrome or insulin resistance [15]. Furthermore, it is proposed that endothelial dysfunction itself may induce insulin resistance [16]. Thus, endothelial dysfunction is closely linked to pathogenesis of metabolic syndrome and it reflects multiple factors that contribute to CVD events in metabolic syndrome. In this study, we tested whether RLP-C levels are a risk factor for coronary artery disease (CAD) and endothelial vasomotor dysfunction in patients with metabolic syndrome. Furthermore, we examined the therapeutic effects of bezafibrate and atorvastatin on RLP-C levels and endothelial vasomotor dysfunction in a subgroup of the study patients.

2. Methods

2.1. Study patients

This study enrolled 132 consecutive patients with the metabolic syndrome and CAD who underwent cardiac catheterization for chest pain or ischemic changes detected by electrocardiogram. All patients had angiographic evidence of organic diameter stenosis of >70% of at least one major coronary artery (single-vessel disease, 48 patients; two-vessel disease, 45 patients; three-vessel disease, 29 patients; left main coronary artery disease, 10 patients). The metabolic syndrome was defined as the presence of three or more of the following abnormalities: waist circumference ≥85 cm in male and ≥90 cm in female (according to The Examination Committee of Criteria for Obesity Disease in Japan [17]), fasting glucose levels ≥110 mg/dL, triglyceride levels ≥150 mg/dL, HDL levels <40 mg/dL in male and <50 mg/dL in female, blood pressure ≥130/85 mmHg.

This study also enrolled 78 consecutive patients with metabolic syndrome but without CAD who underwent cardiac catheterization for atypical chest pain in the hospital during the same period as the patients with CAD. These non-CAD patients had angiographically normal coronary arteries (<10% stenosis) and normal left ventriculography and thereby formed a case control group to evaluate whether RLP-C levels as a risk factor differed between patients with and without CAD.

The baseline characteristics of the study patients are shown in Table 1. This study was conducted in agreement with guidelines approved by the ethics committee at our institution. Written informed consent was obtained from all patients before the study.

2.2. Lipid-lowering therapy

A subgroup of the consecutive study patients with metabolic syndrome and CAD ($n=40$) were randomly assigned to 4 weeks of oral atorvastatin (10 mg/day) or bezafibrate (400 mg/day). All of the patients were blinded to the content of the tablets. They were advised to adhere to their usual diet and lifestyle throughout the 4-week treatment period. Measurements of vasomotor function in the brachial artery and blood sampling were performed in the same manner after an overnight fast on the same morning before and at the end (4 weeks) of treatment. All medications were withdrawn 12 h before the measurements.

Table 1
Characteristics of study patients

	Without CAD ($n=78$)	With CAD ($n=132$)	p-value
Age (years)	64 ± 3.9	64 ± 3.8	NS
Gender male (%)	78	78	NS
waist (cm)	91 ± 4	92 ± 4	NS
Hypertension (%)	58	61	NS
DM (%)	64	70	NS
BMI (kg/m^2)	26.4 ± 2.9	26.6 ± 3.2	NS
Triglyceride (mg/dL)[a]	176 (149, 202)	184 (146, 241)	0.02
HDL-C (mg/dL)[a]	46 (38, 57)	43 (37, 51)	NS
LDL-C (mg/dL)	136 ± 22	136 ± 26	NS
RLP-C (mg/dL)[a]	5.9 (4.0, 7.4)	7.0 (5.1, 9.7)	<0.0001
HOMA-IR	2.3 ± 1.1	2.4 ± 1.1	NS
FFA (mg/dL)	561 ± 210	563 ± 287	NS
hsCRP (mg/dL)[a]	0.07(0.03, 0.12)	0.16 (0.08, 0.40)	<0.0001
IL-6 (pg/mL)	2.3 ± 1.3	3.5 ± 2.4	0.0002
TNF-α (pg/mL)	2.4 ± 1.3	2.8 ± 0.9	0.0002
Leptin (ng/mL)	11.3 ± 5.1	13.3 ± 5.9	0.01
FMD (%)	5.0 ± 1.6	3.8 ± 1.6	<0.0001

[a] Expressed as the median value (interquartile range). Other data are expressed as the mean value ±S.D. or frequency (%) of patients. Waist, waist circumference; DM, diabetes mellitus; BMI, Body mass index; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; FFA, free fatty acid; hsCRP, high sensitive C-reactive protein; IL-6, Interleukin 6; TNF-α, Tumor necrosis factor-α; RLP-C, remnant-like lipoprotein particles-cholesterol; FMD, flow-mediated dilatation of brachial artery.

2.3. Measurements of flow-mediated dilation (FMD) in brachial artery

Vasodilator responses in the brachial arteries were measured by use of B-mode ultrasound images with a 7.5-MHz linear array transducer (HP-5500, Phillips Corp., Tokyo, Japan) as validated previously by us as well as others [18,19]. Measurements were performed by two observers who were blinded to the study protocol and the subject grouping. The brachial artery was scanned in the antecubital fossa in a longitudinal fashion. Optimal brachial artery images were obtained between 1 and 5 cm above the antecubital crease. This location was marked, and all subsequent images were obtained at the same location. The exact distance of the measured point of the skin surface from the antecubital crease was recorded in each study subject to ensure that the same location of the brachial artery was measured at each time point. Gain setting

was optimized at the beginning of the study and was kept constant throughout the recording period. After baseline measurements of the diameter and flow velocity in the brachial artery, a blood pressure cuff was placed around the forearm and inflated with a pressure of 250–300 mmHg for 5 min, and then released. Diameter measurements during reactive hyperemia were taken 45–90 s after cuff deflation. Then, sublingual nitroglycerin (300 μg) was administered, and 3 min later the measurements were repeated. Images were recorded on a super-VHS videocassette recorder (model BR-S601M, Victor Corp., Tokyo, Japan), and brachial arterial diameters were measured from the tape with ultrasonic calipers, as described previously [18,19]. The response of the vessel diameter to reactive hyperemia (flow-mediated dilation; FMD) and nitroglycerin was expressed as a percentage increase in the diameter from the baseline value. The diameter responses were assessed at three points along a 10-mm length of the artery, and results were averaged. Blood flow was calculated by multiplying the velocity–time integral of the Doppler flow signal by heart rate and the vessel cross-sectional area. Increase in brachial blood flow was calculated as a maximum flow recorded in the first 15 s after cuff deflation and was expressed as a percentage increase in the flow from the baseline value.

2.4. Assays

At the beginning of the study, venous blood was obtained from all patients after a 12-h overnight fast. All patients ate standard Japanese meals (1900 kcal/d, 25% fat, 59% carbohydrate, and 16% protein) the day before blood sampling [8–11]. Serum was stored at 4 °C and was used for assays of lipoproteins and lipids within 3 days after sampling. The plasma and the remaining serum were stored at −80 °C until other assays were performed. RLP was isolated by application of the fasting serum to an immunoaffinity mixed gel that contained anti-apoA-1 and anti-apoB-100 monoclonal antibodies (Japan Immunoresearch Laboratories), according to the method, described in previous reports [8–14]. Levels of HDL-cholesterol, LDL-cholesterol, and triglyceride in fasting serum were measured as described previously [8–11].

Tumor necrosis factor-alpha (TNFα), interleukin (IL)-6, and leptin concentrations in fasting plasma were measured by enzyme linked immunosorbent assay (ELISA) with commercially available kits. High sensitive C-reactive protein (hsCRP) levels in the serum were assayed by rate nephelometry (Dade Behring). The insulin resistance index was assessed by homoeostasis model assessment for insulin resistance (HOMA-IR).

2.5. Statistical analyses

The levels of RLP-C, triglyceride, HDL-C, and hsCRP were not distributed normally, and therefore, these data were expressed as the median and range (25 and 75th percentiles) and were log-transformed when these data were statistically analyzed. The mean value and frequency (Table 1) were compared between two groups using the unpaired *t*-test and Chi-square analysis, respectively. For comparison of lipids and biochemical parameters before and after treatment with bezafibrate or atorbastatin (Table 4), two-way analysis of variance for repeated measures followed by post hoc testing with Sheffe's test was used. The assessment of independent association of risk factors (as independent variables) with CAD was performed by multivariate logistic regression analysis using the independent variables that had a significant difference between patients with and without CAD using an unpaired *t*-test and Chi-square analysis. The following factors were included as categorical variables: high levels of RLP-C (≥5.0 mg/dL according to our previous reports [8–11]); high levels of triglyceride (≥150 mg/dL); high levels of hsCRP (≥0.3 mg/dL; arbitrarily defined as the 75th percentile of the distribution of the levels in the study patients), and high levels of IL-6, TNFα, and leptin (≥2.6 pg/mL, 2.5 pg/mL, and 11 ng/mL, respectively; arbitrarily defined as the 50th percentile of the distribution of the respective levels in the study patients). The assessment of independent correlation of risk factors with FMD was performed by multivariate linear regression analysis using the independent variables that had a significant correlation with FMD in the univariate analysis. Statistical significance was defined as $p < 0.05$. Analyses were assessed in part using StatView 5.0 for Windows (Tokyo, Japan).

3. Results

3.1. Comparisons of risk factors between patients with and without CAD

Risk factor profiles in the study patients are shown in Table 1. The fasting levels of triglyceride, RLP-C, hsCRP, IL-6, TNFα, and leptin were significantly higher in CAD patients with metabolic syndrome compared with non-CAD patients with metabolic syndrome. Comparison of risk factors between the metabolic-syndrome patients with and without CAD using multivariate logistic regression analysis demonstrated that high levels of RLP-C, hsCRP, IL-6, TNFα, and leptin remained independent risk factors for the presence of CAD (Table 2). Moreover, high RLP-C levels had the strongest association with CAD among these covariates. In addition, high RLP-C levels were an independent risk factor for CAD in a subgroup of the patients with normotriglyceridemia (Table 2). It was clear that RLP-C levels in combination with high hsCRP levels had an incremental effect on the risk of CAD (Fig. 1, upper panel).

3.2. Correlation of risk factors with FMD in patients with metabolic syndrome and CAD

FMD was significantly impaired in the patients with CAD as compared with those without CAD, as shown in Table 1.

Table 2
Association of lipids and biochemical parameters with coronary artery disease

	All patients		Patients with normo-triglyceridemia	
	Relative risk (95% CI)	*p*-value	Relative risk (95% CI)	*p*-value
RLP-C (≥5.0 mg/dL)	7.0 (2.7–17)	<0.0001	6.8 (2.6–17)	<0.0001
Triglyceride (≥150 mg/dL)	0.4 (0.2–0.9)	NS	–	–
hsCRP (≥0.3 mg/dL)	6.5 (2.6–16)	<0.0001	4.2 (2.0–8.7)	0.0001
IL-6 (≥2.6 pg/mL)	2.5 (1.3–5.2)	<0.001	2.5 (1.2–5.2)	0.01
TNFα (≥2.5 pg/mL)	3.6 (1.7–7.7)	0.001	3.2 (1.5–6.8)	<0.01
Leptin (≥11 ng/mL)	2.1 (1.1–4.4)	<0.05	1.9 (0.9–4.0)	NS

CI, confidence interval. Other abbreviations as in Table 1.

FMD had a significant and inverse correlation with levels of RLP-C, triglyceride, hsCRP, IL-6, TNFα, and HOMA-IR in patients with metabolic syndrome and CAD using univariate linear regression analysis (Table 3). Further, FMD also had a positive correlation with HDL-C levels. FMD was comparable between patients with and without smoking status, diabetes, hypertension, or male sex as categorical risk factors (data not shown). Using multivariate linear regression analysis, FMD had an independent and inverse correlation with levels of RLP-C, triglyceride, and hsCRP after adjustment for levels of HDL-C, IL-6, and TNFα, and HOMA-IR (Table 3). Furthermore, RLP-C levels had a stronger association with FMD than triglyceride and hsCRP levels. It was clear that high RLP-C levels in combination with high hsCRP levels had an incremental effect on the risk of endothelial vasomotor dysfunction (Fig. 1, lower panel). Dilator response to nitrate was not correlated with either RLP-C levels or hsCRP levels ($r=0.03$, $p=$NS, $r=0.01$, and $p=$NS, respectively).

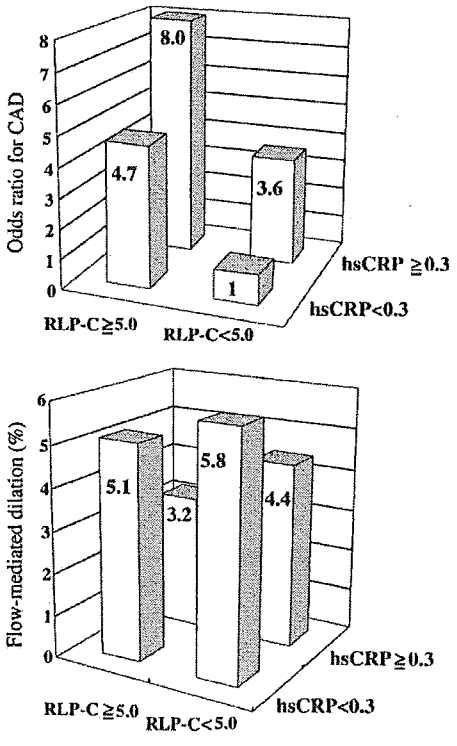

Fig. 1. Upper panel, incremental effect on odds ratios for CAD of the combination of higher levels of RLP-C and hsCRP. Lower panel, incremental effect on FMD of the combination of higher levels of RLP-C and hsCRP.

3.3. *Effects of treatment with bezafibrate or atorvastatin on FMD and other parameters in patients with metabolic syndrome and CAD*

Before treatment, there was not significant difference in FMD, lipid levels, and values of other parameters tested between patients treated with bezafibrate and atorvastatin (Table 4). Treatment for 4 weeks with bezafibrate or atorvastatin significantly decreased levels of RLP-C, triglyceride, LDL-C, hsCRP, IL-6, TNFα, and HOMA-IR, and increased HDL-C levels (Table 4). LDL-C levels had a greater decrease in the atorvastatin group than the bezafibrate group, while the other parameters had comparable changes from pretreatment values between the two treatment groups (Table 4). Also, bezafibrate and atorvastatin significantly improved FMD to a comparable degree after 4 weeks of therapy (Table 4). Using univariate linear regression analysis, the changes in FMD from baseline (post-treatment value minus pre-treatment

Table 3
Univariate and multivariate linear regression analyses; correlation of FMD with lipid and biochemical parameters and components of metabolic syndrome

	Univariate analysis		Multivariate analysis	
	r	*p*-value	Standardized regression coefficient	*p*-value
RLP-C	−0.64	<0.0001	−0.46	<0.0001
Triglyceride	−0.51	<0.0001	−0.3	0.002
hsCRP	−0.59	<0.0001	−0.26	0.002
HDL-C	0.39	0.02	0.07	NS
IL-6	−0.29	0.0002	−0.08	NS
TNFα	−0.25	0.001	−0.07	NS
HOMA-IR	−0.26	0.001	−0.12	NS
Leptin	−0.02	NS	–	–
Age	−0.13	NS	–	–
Waist circumference	−0.1	NS	–	–
BMI	0.02	NS	–	–
Systolic BP	0.01	NS	–	–

r, regression coefficient; FMD, flow-mediated dilation; BP, blood pressure. Other abbreviations as in Table 1.

Table 4
Changes of lipids and biochemical markers after treatment with bezafibrate or atorvastatin

	Bezafibrate ($n=20$)		Atorvastatin ($n=20$)	
	Before	After treatment	Before	After treatment
FMD (%)	4.02 ± 2.8	5.9 ± 3.4*	4.1 ± 2.9	6.0 ± 3.4*
RLP-C (mg/dL)[a]	9.2 (7.0, 12)	5.2 (4.6, 6.5)**	9.1 (7.2, 10.6)	5.3 (4.5, 6.7)**
Triglyceride (mg/d)[a]	200 (155, 253)	136 (80, 172)**	202 (171, 251)	141 (101, 163)**
HDL-C (mg/dL)[a]	45 (38, 53)	56 (43, 62)**	46 (37, 55)	51 (43, 61)**
LDL-C (mg/dL)	133 ± 24	119 ± 31**	139 ± 34	85 ± 23[b,**]
hsCRP (mg/Dl)[a]	0.23(0.12, 0.30)	0.09 (0.05, 0.1)**	0.3 (0.13, 0.40)	0.09 (0.03, 0.28)**
IL-6 (pg/mL)	2.6 ± 1.5	2.3 ± 1.1*	2.6 ± 1.7	2.2 ± 1.1*
TNFα(pg/mL)	2.31 ± 0.71	2.07 ± 0.44**	2.32 ± 0.70	2.02 ± 0.67**
HOMA-IR	2.2 ± 1.6	1.5 ± 0.7*	2.13 ± 0.9	1.68 ± 0.6*

FMD, flow mediated vasodilatation. Other abbreviations as in Table 1.
[a] Expressed as the median value (interquartile range). Other data are expressed as the mean value ± S.D.
[b] $p<0.01$ versus after treatment with bezafibrate.
* $p<0.05$.
** $p<0.01$ versus respective values before treatment.

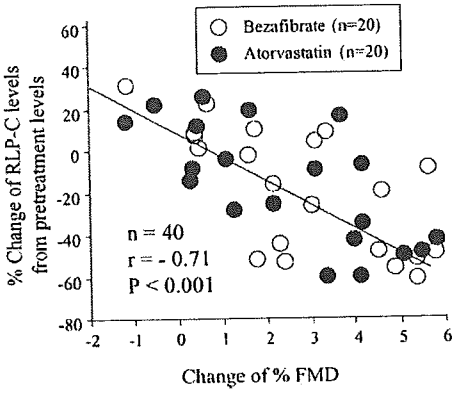

Fig. 2. Correlation of the change in FMD with the % change in RLP-C levels from pre-treatment values after treatment with bezafibrate (open circles) or atorvastatin (closed circles). The changes in FMD were calculated as the differences between pre-treatment and post-treatment values (post-treatment values minus pre-treatment values).

value) after the treatment with the lipid-lowering medications had a significant association with the % changes in levels of RLP-C, triglyceride, HOMA-IR, hsCRP, and IL-6 from their pre-treatment values after treatment (Fig. 2 and Table 5). Using multivariate linear regression analysis, the % changes in RLP-C levels had a significant and independent association with the changes in FMD after adjustment for the drugs assigned and the % changes in levels of triglyceride, HOMA-IR, hsCRP, and IL-6 (Table 5).

4. Discussion

This study showed that high RLP-C levels were the strongest risk factor for CAD and the impaired FMD in patients with metabolic syndrome independently of the covariates including the components of metabolic syndrome and the proinflammatory markers levels. Furthermore, this study showed that treatment with atorvastatin or bezafibrate induced rapid improvement of FMD in patients with metabolic syndrome. Also, treatment with atorvastatin or bezafibrate improved dyslipidemia including high RLP-C levels and decreased proinflammatory markers levels. The reduction of RLP-C levels after treatment with the lipid-lowering medications had a strong association with the improvement of FMD independently of drugs assigned and changes in levels of triglyceride, HOMA-IR, hsCRP, and IL-6. On the other hand, neither RLP-C levels nor other risk factors was correlated with dilator response to nitrates, an endothelium-independent dilation. Therefore, measurement of RLP-C is useful for the assessment of therapeutic effects on endothelial vasomotor dysfunction in metabolic syndrome.

We and others have shown that RLP-C levels are increased and predict future coronary events in patients with CAD and type II DM or insulin resistance [11]. Based on the mechanisms as described in previous reports, [6,7,11] increased flux

Table 5
Univariate and multivariate linear regression analyses; correlation between changes in FMD and lipids and biochemical parameters from baseline values after treatment

	Univariate analysis		Multivariate analysis	
	r	p-value	Standardized regression coefficient	p-value
RLP-C	−0.71	<0.0001	−0.66	<0.0001
Triglyceride	−0.46	<0.05	−0.31	NS
HOMA-IR	−0.41	<0.05	−0.2	NS
hsCRP	−0.55	<0.01	−0.58	<0.001
IL-6	−0.47	<0.01	−0.3	NS
TNFα	−0.3	NS	–	–
LDL-C	−0.13	NS	–	–
HDL-C	0.13	NS	–	–

r, regression coefficient. Other abbreviations as in Tables 1–3. Multivariate regression analysis included only the covariates that related to changes in FMD in the univariate analysis. Changes in FMD, post-treatment FMD minus pre-treatment FMD.

of free fatty acids from the periphery to the liver might cause hepatic production and secretion of triglyceride-rich VLDL, leading to increase in circulating levels of remnant lipoproteins in patients with metabolic syndrome. However, the causes of remnant lipoproteinemia in the metabolic syndrome are multifactorial and linked with each other and not simply a function of increased free fatty acid and flux to the liver. For example, a proinflammatory state intimately connects with dyslipidemia in metabolic syndrome [1,5]. Elevated levels of TNFα and IL-6, independent risk factor for CAD in the present patients with metabolic syndrome are known to increase triglyceride levels, [20,21] which could contribute to remnant lipoproteinemia. Furthermore, the present study demonstrated that high levels of hsCRP also were an independent risk factor for endothelial vasomotor dysfunction and CAD. When we categorized patients according to RLP-C levels and hsCRP levels at baseline, higher levels of RLP-C and hsCRP were additive in their effect on the risk of endothelial vasomotor dysfunction and CAD in patients with metabolic syndrome. Taken together, these results are compatible with previous data that chronic subclinical inflammation is an important factor in the pathogenesis of metabolic syndrome [22].

The present study showed that high RLP-C levels were an independent risk factor for CAD even in a subgroup of the patients with metabolic syndrome and normo-triglyceridemia. Furthermore, high RLP-C levels had a stronger association with endothelial vasomotor dysfunction than high triglyceride levels. Although RLP levels are closely and positively correlated with triglyceride levels, [8] the present data indicate that high RLP-C levels are a better marker of dyslipidemia than hyper-triglyceridemia in this syndrome.

As described in previous reports, [15,16] FMD is likely to be lower in the present non-CAD patients with metabolic syndrome as compared with control patients without metabolic syndrome and CAD in our database (5.0 ± 1.6 versus 7.7 ± 1.8, respectively) [18,19]. We showed recently that RLP increased the susceptibility of the coronary endothelium to oxidative stress, [8,13] leading to inhibition of nitric oxide-mediated dilation of human coronary arteries [8]. Furthermore, we showed that RLP upregulated the expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and tissue factor in cultured human endothelial cells [12]. In addition, there is evidence that RLP enhances platelets aggregation [23]. These proatherothrombogenic effects of RLP may explain the association of high RLP-C levels with the increased prevalence of CAD and endothelial dysfunction in metabolic syndrome. In this regard, high RLP-C levels could be a distinct patho-physiological feature of metabolic syndrome, and thus measurement of RLP-C is useful for identification of high-risk populations. Measurement of remnant lipoproteins has been difficult because of the heterogeneous nature of these macromolecules. Traditional methods using ultracentrifugation or agarose gel or low-concentration polyacrylamide gel electrophoresis are complex and time-consuming and therefore not applicable for clinical use. The immunoseparation method used in the present study has been shown by us and other investigators to be both simple and reliable and therefore useful for assessing and monitoring CAD risk.

The present study showed that bezafibrate and atorvastatin, different types of lipid-lowering drugs, exerted beneficial effects on FMD, levels of RLP-C and triglyceride, HOMA-IR, and proinflammatory markers with comparable degree except for LDL-C levels. The beneficial effect on vasomotor function is consistent with a previous report, [24] but this previous study showed that the improvement of FMD was not correlated with changes in lipids profiles after treatment with fenofibrate or atorvastatin, though remnant lipoproteins levels were not assessed. Several reports have shown that both statin and fibrate are capable of improving insulin sensitivity through a reduction of triglyceride levels or their pleiotropic effects [25–27]. Thus, the reduction in HOMA-IR after treatment with atorvastatin and bezafibrate may be partly mediated through direct or indirect favorable effects of atorvastatin and bezafibrate on insulin sensitivity. It remains undetermined which of statins and fibrates, first-line lipid-lowering drugs, are more useful for preventing CVD in metabolic syndrome.

In conclusion, elevated levels of RLP-C are a risk factor for endothelial dysfunction and CAD in metabolic syndrome. Measurement of RLP-C is useful for assessment of CAD risk and therapeutic effects in metabolic syndrome.

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Increased Ambulatory Pulse Pressure Is a Strong Risk Factor for Coronary Endothelial Vasomotor Dysfunction

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OBJECTIVES This study was aimed to determine the relationship between pulse pressure (PP) and coronary vasomotor dysfunction, a predictor of coronary events.

BACKGROUND Pulse pressure is a strong risk factor for coronary artery disease (CAD). However, the mechanisms by which an increase in PP affects the pathogenesis of CAD are unclear.

METHODS Ambulatory blood pressure (BP) monitoring for 24 h was performed in 103 consecutive patients with normal coronary angiograms (51 hypertensive and 52 normotensive; age 42 to 70 years). The relationship between changes in coronary arterial diameter and blood flow during an intracoronary infusion of acetylcholine (ACh) (5, 10, 50 μg/min), and BP parameters, and other traditional risk factors was evaluated using univariate and multivariate linear regression analyses.

RESULTS With multivariate analyses, the 24-h PP showed an inverse correlation with the epicardial coronary dilator response to ACh independently of other covariates including age, smoking, and 24-h systolic BP in normotensive as well as hypertensive patients. Furthermore, multivariate analysis showed that the 24-h PP was inversely and independently correlated with the increase in coronary blood flow in response to ACh. The dilator response of epicardial coronary arteries to nitrate was not significantly correlated with 24-h PP.

CONCLUSIONS Increased 24-h PP is independently associated with endothelial vasomotor dysfunction in conduit and resistance coronary arteries irrespective of the presence of hypertension. Increased ambulatory PP may have an intimate relation to coronary endothelial vasomotor dysfunction. (J Am Coll Cardiol 2005;45:1461–6) © 2005 by the American College of Cardiology Foundation

Pulse pressure (PP), calculated as the difference between systolic blood pressure (SBP) and diastolic blood pressure (DBP), has been previously reported to be a stronger cardiovascular risk factor than SBP alone, especially in elderly hypertensive patients (1). Even in normotensive subjects, an increase in PP remains a powerful and independent predictor of cardiovascular risk, particularly for myocardial infarction (2). However, the underlying mechanisms by which an increase in PP plays a role in pathogenesis of coronary artery disease remain unclear. A recent report (3) showed that an increase in PP was associated with endothelial vasomotor dysfunction, an independent predictor of future coronary events, in the resistance vessels downstream from the brachial artery in hypertensive patients. However, there is no information on the effects of PP on endothelial vasomotor functions in human coronary arteries in normotensive or hypertensive patients. Furthermore, the relationship between PP and endothelial vasomotor function in the coronary arteries may not be similar to the brachial artery because of the predominant role of DBP in the coronary circulation. Thus, the objective of this study was to determine whether an increase in PP might be associated with endothelial vasomotor dysfunction in the conduit and resistance vessels in the coronary circulation in both normotensive and hypertensive subjects.

METHODS

Study patients. Study subjects consisted of a consecutive series of 103 patients. Characteristics of the study subjects are shown in Table 1. They underwent diagnostic coronary angiography for atypical chest pain (95 subjects) or ST-segment depression at rest or during exercise without chest pain (8 subjects) in Yamanashi University Hospital between January 2002 and January 2004. They fulfilled all of the following inclusion criteria: 1) angiographically normal coronary arteries (<5% narrowing after nitrate administration) and no coronary spasm (<50% decrease in epicardial

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Abbreviations and Acronyms

ACh = acetylcholine
BP = blood pressure
DBP = diastolic blood pressure
HR = heart rate
NO = nitric oxide
PP = pulse pressure
SBP = systolic blood pressure

coronary diameter from baseline and neither chest pain nor ischemic electrocardiographic change) after the intracoronary infusion of acetylcholine (ACh); 2) normal left ventriculography; 3) no left ventricular hypertrophy, verified by both electrocardiography and echocardiography; and 4) no history of myocardial infarction, congestive heart failure, valvular heart disease, secondary hypertension, stroke, renal dysfunction (serum creatinine concentration >2.0 mg/dl) or other serious diseases. All medications that could have affected coronary vasomotor reactivity and blood pressure (BP) were withdrawn ≥3 days before the study. Hypertension was defined according to Joint National Committee on Prevention Detection, Evaluation, and Treatment of High Blood Pressure-VI criteria (4): the averaged values of two or more BP measurements obtained on at least two separate occasions were >140 mm Hg SBP or >90 mm Hg DBP, with waking ambulatory BP measurements >135/85 mm Hg or sleeping ambulatory BP measurements >120/75 mm Hg. Written informed consent was obtained from all study subjects before the study. The study was approved by the ethics committee at our institution.

Protocol for coronary angiography. After baseline angiography, incremental doses of ACh (5, 10, and 50 μg/min) were infused directly into the left coronary artery through the Judkins catheter for 2 min with a 5-min interval between each dose (5). Hemodynamic measurements and coronary angiography were repeated before and during each of the ACh infusions. After an additional 15 min, intracoronary injection of isosorbide dinitrate (1 mg) was performed; 2 min after that, coronary angiography was performed in multiple projections in all study subjects.

Ambulatory BP measurements. Systolic BP, DBP, PP, and heart rate (HR) during daily activities were measured every 30 min for 24 h, by the oscillometric method, using a noninvasive ambulatory BP monitoring system (TM-2425, A&D, Tokyo, Japan) (6). The daytime and nighttime mean values of SBP, DBP, PP, and HR during the 24-h period were analyzed after reviewing the patients' diaries. We defined daytime as the period from the time they awoke to the time they went to sleep, and nighttime as the period during which they were sleeping (7). The daytime, nighttime, and 24-h SBP, DBP, PP, and HR were the averages of all of the values obtained at 30-min intervals. Non-dipper hypertension was defined by the absence of the fall (>10%) in the nighttime mean SBP, and/or in DBP from the respective daytime values (7).

Quantitative coronary angiography and the measurement of coronary blood flow. A quantitative coronary angiographic study was performed in all of the study subjects with the Judkins technique in the morning when the patients were fasting, in the same manner as described previously (5). Measurement of luminal diameter of the left anterior descending coronary artery at the midsegment was performed quantitatively by use of a computer-assisted coronary angiographic analysis system (Cardio 500, Kontron Instruments, Munich, Germany) by two observers blinded to the study protocol. Responses of the coronary artery diameter to infusions of ACh and nitrates were expressed as percent changes from baseline diameter measured on angiograms taken just before each infusion.

Blood flow velocity was measured in a subgroup of 56 consecutive subjects using a 0.014-inch wire equipped with a Doppler crystal at its tip (Flow Wire, Cardiometrics, Mountain View, California), which was advanced through the Judkins catheter and carefully positioned in a straight proximal segment of the left anterior descending coronary artery to obtain a stable flow velocity signal (5). The stable peak flow velocity signals at baseline and during a 2-min

Table 1. Study Patients' Characteristics

	All Patients (n = 103)	Hypertensive Patients (n = 51)	Normotensive Patients (n = 52)
Age (yrs)	62 ± 11	63 ± 11	61 ± 12
Male (%)	45	45	44
Body mass index (kg/m²)	24 ± 4	25 ± 5	24 ± 3
Smoking (%)	45	49	41
Total cholesterol (mg/dl)	205 ± 36	206 ± 39	203 ± 33
Diabetes mellitus (%)	26	30	22
Hypertension (%)	50	—	—
Non-dipper (%)	31	62*	—
Ambulatory daytime PP (mm Hg)	50 ± 11	54 ± 13	47 ± 8†
Ambulatory daytime SBP (mm Hg)	127 ± 17	140 ± 16*	119 ± 12*†
Baseline coronary diameter (mm)	3.3 ± 0.8	3.4 ± 1.0	3.2 ± 0.6
Baseline coronary flow (ml/min)	83 ± 39	85 ± 40	80 ± 39

Data are expressed as mean ± SD and percentage. *$p < 0.05$ vs. all patients; †$p < 0.05$ vs. hypertensive patients.
PP = pulse pressure; SBP = systolic blood pressure.

infusion of ACh at doses of 5 and 10 μg/min were used for the analysis (Flow Map, Cardiometrics). Coronary blood flow (ml/min) was estimated from coronary blood flow velocity and arterial diameter by the following formula: 0.5 × averaged peak velocity (cm/min) × cross-sectional area (cm^2). The response of coronary blood flow to intracoronary infusions of ACh was expressed as a percentage change from the baseline blood flow just before ACh infusion.

Statistical analysis. Results are expressed as the mean ± SD or percentage. The mean values of continuous variables were compared between the two groups using an unpaired *t* test and frequencies were compared by chi-square analysis. Comparison of continuous variables among more than three groups was performed using one-way analysis of variance. Linear regression analysis was used to determine the relationship between the coronary responses and all continuous variables. Multivariate linear regression analyses were also used to determine the relationship between coronary responses and 24-h ambulatory PP; independent covariates included any continuous variable that was significantly correlated with the coronary responses in the univariate analysis. In addition, the multivariate analysis also included any categorical risk factor that led to a significant difference in coronary responses when patients with and without the traditional risk factors were compared using an unpaired *t* test. The categorical variables were coded using the following dummy variables: 0 for the absence of the risk factor; or 1 for the presence of the risk factor. When correlation between coronary flow response and risk factors was analyzed in the multivariate analysis, only data from all patients were used because there were too few patients tested for coronary flow response in the various subgroups. A confidence level of $p < 0.05$ was considered statistically significant. Analyses were partially assessed using StatView 5.0 (SAS Institute, Cary, North Carolina).

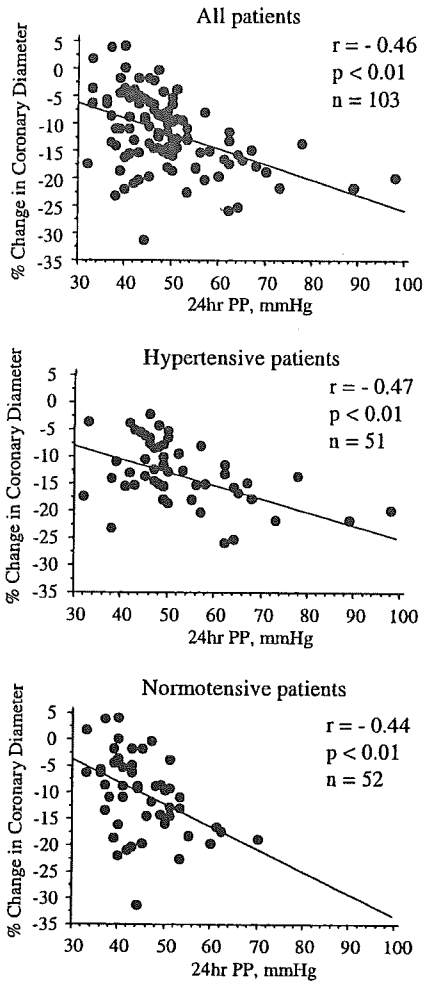

Figure 1. Correlations of 24-h ambulatory pulse pressure (PP) with percent (%) changes of epicardial coronary dilator responses to acetylcholine (50 μg/min) in all the study patients, hypertensive patients, and normotensive patients.

RESULTS

Comparisons of clinical characteristics and parameters among all study patients, hypertensive patients, and normotensive patients. None of the clinical characteristics or parameters except the BP parameters was significantly different among the three groups, all patients, hypertensive patients, and normotensive patients (Table 1).

Correlations of epicardial coronary diameter responses with clinical characteristics and BP parameters. Intracoronary infusion of ACh constricted the coronary arteries in a majority of patients and dilated the arteries in a small number of patients, resulting in an overall constrictor response. Using univariate linear regression analysis, age and 24-h ambulatory PP and SBP had a significant and inverse correlation with the dilator responses of epicardial coronary arteries to ACh at a dose of 50 μg/min in all patients (Fig. 1, Table 2). Age and ambulatory PP and SBP also showed a significant correlation in the subgroup of normotensive patients, as shown in Table 2. As shown in Table 3, smokers had an impaired dilation or an enhanced constriction of epicardial coronary arteries to ACh as compared with nonsmokers in all three of the study groups (Table 3). Using multivariate linear regression analysis after adjustment for age, smoking status, and ambulatory SBP as covariates, 24-h ambulatory PP remained significantly and inversely correlated with the coronary diameter response to ACh at a dose of 50 μg/min in all patients, the hypertensive patients, and the normotensive patients (Table 4). Ambulatory PP also was independently correlated with the diameter responses to ACh at 5 and 10 μg/min in all patients as well as normotensive patients (standardized regression coefficient, 5 μg/min; −0.47 and −0.53, respectively; $p < 0.05$ in both; 10 μg/min; −0.44 and −0.47, respectively; $p < 0.05$ in both). The dilator response to nitrates was not significantly correlated with 24-h ambulatory PP in either all patients or just the normotensive patients ($r = -0.1$, p = NS; $r = -0.12$, p = NS, respectively).

Correlation of coronary flow responses with clinical characteristics and BP parameters. Coronary blood flow was increased in response to ACh infusion in all patients

Table 2. Relationships of Coronary Risk Factors (Continuous Variables) With the Percent (%) Changes in Diameter of Epicardial Coronary Arteries and Coronary Blood Flow Response to ACh Using Univariate Linear Regression Analysis

	% Change in Epicardial Diameter (ACh 50 μg/min)			% Change in Coronary Flow (ACh 5 μg/min)		
	All Patients (n = 103)	Hypertensive Patients (n = 51)	Normotensive Patients (n = 52)	All Patients (n = 56)	Hypertensive Patients (n = 29)	Normotensive Patients (n = 27)
Age	−0.50†	−0.44†	−0.53†	−0.31*	0.24	−0.41*
BMI	−0.08	−0.01	−0.10	0.04	−0.06	−0.13
Total cholesterol	0.06	0.09	0.04	−0.23	−0.33	−0.08
24-h PP	−0.46†	−0.47†	−0.44†	−0.40†	−0.38*	−0.44*
24-h SBP	−0.40†	−0.35*	−0.43†	−0.36*	−0.39*	−0.25
24-h DBP	−0.18	−0.03	−0.23	−0.18	−0.20	0.02
Office PP	−0.28	−0.27	−0.30	−0.29	−0.34	−0.17
Office SBP	−0.26	−0.20	−0.19	−0.29	−0.36	−0.19
Office DBP	0.04	0.22	0.23	−0.07	0.22	−0.06

Data are expressed as regression coefficient. *$p < 0.05$; †$p < 0.01$.
ACh = acetylcholine; BMI = body mass index; DBP = diastolic blood pressure; PP = pulse pressure; SBP = systolic blood pressure.

studied. Using univariate linear regression analysis, age and ambulatory 24-h PP and SBP had a significant and inverse correlation with percent increase of coronary flow in response to ACh at doses of 5 μg/min in all patients (Fig. 2, Table 2). Age and ambulatory PP also had a significant correlation in the subgroup of the normotensive patients (Table 2). Diabetic patients had an impaired increase of coronary flow to ACh as compared with nondiabetic patients in all of the three study groups (Table 3). In all of the patients, 24-h ambulatory PP remained significantly and inversely correlated with the percent increase of flow in response to ACh at doses of 5 μg/min using multivariate linear regression analysis after adjustment for age, ambulatory SBP, and diabetes (these covariates were significantly related to the flow response in the univariate linear regression analysis or the unpaired *t* test) (Table 4). Also, the independent and inverse correlation of 24-h ambulatory PP with the percentage increase of flow in response to ACh at doses of 10 μg/min was observed in all of the patients (standardized regression coefficient = −0.45, $p < 0.05$).

DISCUSSION

The present study assessed the relation between PP and endothelial vasomotor function in human coronary arteries. Multivariate analyses indicated that increased ambulatory PP had a significant and independent correlation with abnormal vasomotor reactivity in both the conduit and resistance vessels in the coronary circulation, as demonstrated by impaired dilation or enhanced constriction of epicardial coronary arteries and by the impairment of the coronary blood flow increase in response to an intracoronary

Table 3. Comparisons of Percent (%) Changes in Diameter of Epicardial Coronary Arteries and Coronary Blood Flow Response to ACh Using Categorical Variables to Classify Patients

	% Change in Epicardial Diameter (ACh 50 μg/min)					
	All Patients (n = 103)		Hypertensive Patients (n = 51)		Normotensive Patients (n = 52)	
	Presence	Absence	Presence	Absence	Presence	Absence
Male	−13 ± 7	−10 ± 6	−15 ± 5	−12 ± 6	−12 ± 8	−9 ± 7
Smoking	−14 ± 6	−9 ± 6*	−15 ± 5	−11 ± 6*	−13 ± 7	−8 ± 7*
Diabetes	−14 ± 5	−11 ± 7	−15 ± 5	−12 ± 6	−11 ± 5	−10 ± 8
Hypertension	−13 ± 6	−10 ± 8				
Non-dipper hypertension			−12 ± 6	−14 ± 5		
	% Change in Coronary Flow (ACh 5 μg/min)					
	All Patients (n = 56)		Hypertensive Patients (n = 29)		Normotensive Patients (n = 27)	
	Presence	Absence	Presence	Absence	Presence	Absence
Male	67 ± 58	59 ± 47	45 ± 45	58 ± 47	89 ± 64	59 ± 49
Smoking	63 ± 57	62 ± 48	45 ± 45	59 ± 46	83 ± 65	65 ± 51
Diabetes	21 ± 35	77 ± 49*	24 ± 39	73 ± 39*	11 ± 10	80 ± 56*
Hypertension	53 ± 46	72 ± 57				
Non-dipper hypertension			51 ± 50	54 ± 39		

Data are expressed as mean ± SD. *$p < 0.05$ vs. presence of respective factors.
ACh = acetylcholine.

Table 4. Multiple Linear Regression Analysis for the Association of Risk Factors With Relative Changes in Epicardial Coronary Diameters and Coronary Flow Response to ACh

	% Change in Epicardial Diameter (ACh 50 μg/min)			% Change in Coronary Flow (ACh 5 μg/min)
	All Patients (n = 103)	Hypertensive Patients (n = 51)	Normotensive Patients (n = 52)	All Patients (n = 56)
Age	−0.45†	−0.34*	−0.51†	−0.14
24-h PP	−0.36*	−0.50†	−0.36*	−0.50†
Smoking	−0.42†	−0.39*	−0.54†	
Diabetes				−0.45†
24-h SBP	−0.19	0.09	−0.05	−0.22

Data are expressed as standardized regression coefficient. *$p < 0.05$; †$p < 0.01$.
ACh = acetylcholine; PP = pulse pressure; SBP = systolic blood pressure.

infusion of ACh. The epicardial coronary diameter responses to nitrates were not significantly correlated with ambulatory PP. Thus, the present results indicate that an increase in ambulatory PP has an independent association with endothelium-dependent vasomotor dysfunction in conduit and resistance coronary vessels in the coronary circulation.

The present study further showed that increased PP also had a significant and independent association with epicardial coronary vasomotor dysfunction in a subgroup of normotensive patients. This may be related to previous findings (2) that an increase in PP is a strong predictor of cardiovascular disease, especially myocardial infarction, independently of other BP parameters. However, prospective studies are required to determine the value of ambulatory PP for the prediction of future cardiac events in patients with preclinical hypertension in order to confirm the results of the present study. In contrast to the usefulness of ambulatory PP in the present study, office PP did not have a significant association with coronary endothelial vasomotor dysfunction. This is consistent with previous reports (1,8,9) that found 24-h ambulatory PP monitoring superior to office PP measurement for predicting cardiovascular disease because ambulatory PP may more accurately reflect the dynamic interaction between the heart and the central stiff arteries during all of the patient's activities.

We and others (10,11) have previously shown that coronary vasomotor regulation largely depends on endothelial nitric oxide (NO) activity. Thus, the decrease in coronary endothelial NO activity may be the underlying mechanism for the coronary endothelial vasomotor dysfunction in the present patients with increased ambulatory PP. The decrease in arterial NO might cause coronary artery spasm (12) and induction of various proatherothrombogenic molecules in the arterial walls (13), resulting in a high incidence of myocardial infarction.

Pulse pressure is largely determined by three hemodynamic factors: arterial stiffness, stroke volume, and wave reflections (14). Among these factors, an increase in arterial stiffness most importantly contributes to the effects of an elevated PP on the risk of cardiovascular disease. An increase in extracellular matrix formation causes arterial stiffness, especially in central arteries, and it might largely contribute to the increase in PP in patients with age and hypertension (15). Angiotensin-converting enzyme inhibitors and angiotensin receptor blockers attenuate extracellular matrix formation in addition to reducing SBP (15). Therefore, these drugs could reduce arterial stiffness, thereby effectively reducing PP as well as SBP.

In conclusion, increased 24-h ambulatory PP has a strong

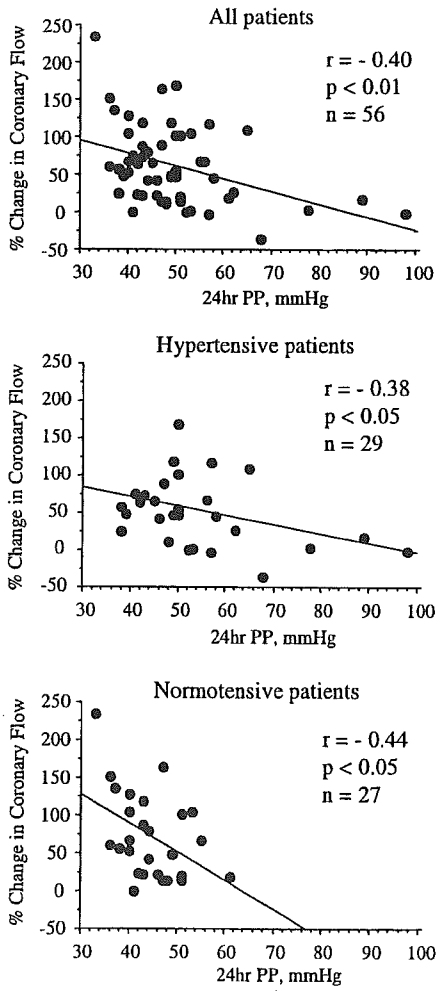

Figure 2. Correlations of 24-h ambulatory pulse pressure (PP) with percent (%) changes of coronary blood flow responses to acetylcholine (5 μg/min) in all the study patients, hypertensive patients, and normotensive patients.

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and independent association with endothelial vasomotor dysfunction in the conduit and resistance vessels in the coronary circulation in normotensive as well as hypertensive patients. Increased ambulatory PP may have an intimate relation to coronary endothelial vasomotor dysfunction.

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Delayed In Vivo Catabolism of Intermediate-Density Lipoprotein and Low-Density Lipoprotein in Hemodialysis Patients as Potential Cause of Premature Atherosclerosis

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Objective—Premature cardiovascular disease is the leading cause of death in patients with end-stage renal disease treated by hemodialysis (HD). Low-density lipoprotein (LDL) levels are not generally increased in HD patients, but their LDL metabolism is still poorly understood. We therefore investigated the in vivo metabolism of apoB-containing lipoproteins in two different ethnic populations of HD patients and controls.

Methods and Results—We performed stable isotope kinetic studies using a primed constant infusion of deuterated leucine in 12 HD patients and 13 healthy controls. Tracer/tracee ratio of apoB was determined by means of gas chromatography/mass spectrometry, and the modeling program SAAMII was used to estimate the fractional catabolic rate (FCR) of apoB. Mean LDL-apoB plasma concentrations were almost identical in both groups (HD: 95±30 mg/dL, controls: 91±40 mg/dL), whereas LDL-apoB FCR was 50% lower in HD patients as compared with controls (0.22±0.12 $days^{-1}$ versus 0.46±0.20 $days^{-1}$, $P=0.001$) with concomitantly decreased production rates of LDL. Compared with controls, intermediate-density lipoprotein (IDL)-apoB FCR was 65% lower (2.87±1.02 $days^{-1}$ versus 8.89±4.94 $days^{-1}$, $P=0.014$), accompanied by 1.5-fold higher IDL-apoB levels in HD. Very low-density lipoprotein metabolism was similar in both study groups.

Conclusions—In vivo catabolism of LDL and IDL is severely impaired in HD patients but misleadingly masked by normal plasma cholesterol levels. The resulting markedly prolonged residence times of both IDL and LDL particles might thus significantly contribute to the well-documented high risk for premature cardiovascular disease in HD patients. (***Arterioscler Thromb Vasc Biol.* 2005;25:2615-2622.**)

Key Words: cardiovascular diseases ■ isotopes ■ kidney ■ lipoproteins ■ metabolism

Thirty yeas ago, Lindner and colleagues recognized in their seminal report the excessive risk of cardiovascular disease for hemodialysis (HD) patients.[1] The prevalence and incidence of cardiovascular disease is much higher in HD patients, and current mortality rates are ≈10 to 20 times greater than the general population with rates even higher at young ages.[2] A remarkable number of factors, including dyslipoproteinemia, chronic inflammation, hypertension, oxidative stress, elevated homocysteine, and anemia, that may contribute to this increased frequency of atherosclerotic complications have been identified.[3,4]

HD patients are characterized by a complex plasma dyslipoproteinemic profile.[5] The most notable quantitative abnormalities are elevated plasma triglyceride and very low-density lipoprotein (VLDL) levels with a prevalence of 25% to 75%,[6,7] increased levels of atherogenic intermediate density lipoprotein (IDL)[8] and lipoprotein(a)[9] particles, and decreased high-density lipoprotein (HDL) levels.[10] Interestingly, total and low-density lipoprotein (LDL) cholesterol plasma levels are usually normal or even subnormal in HD patients as compared with healthy controls.[11,12]

In addition to quantitative changes in lipoprotein particles, numerous compositional and qualitative lipoprotein changes have been demonstrated as well. These include accumulation of small dense LDL[13] as well as oxidation, glycation, and carbamylation of LDL. The association of small dense LDL

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with increased risk for cardiovascular disease in the general population has been controversially discussed.[14] The abnormal lipid composition of all lipoprotein classes has been reported to be caused mainly by a combination of an impaired reversed cholesterol transport[11] and lipolytic cascade.[15]

To date, only 2 apoB kinetic studies have been reported in HD patients: Chan et al injected radio-labeled VLDL into HD patients with or without hyperlipidemia and found decreased fractional catabolic rates (FCR) of VLDL- and IDL-apoB (the latter only in hyperlipidemic patients).[16] Unfortunately, the LDL turnover was not investigated in this study. Hörkkö et al injected radio-labeled LDL and observed a decreased LDL-apoB clearance in renal patients treated with peritoneal dialysis but not in HD patients.[17] Overall, the exact underlying metabolic abnormalities of apoB-containing lipoproteins in HD patients are far from clear. LDL-apoB kinetic studies in predialysis patients with chronic kidney disease have yielded controversial results; radiotracer studies reported decreased LDL clearance rates,[18] whereas more recent studies using stable isotopes found unchanged FCR values for LDL-apoB in these patients.[19]

To resolve the apparent discrepancy between an obviously impaired lipoprotein metabolism and normal LDL plasma concentrations in HD patients, we independently studied the in vivo kinetics of apoB-containing particles in Austrian and Japanese HD patients and healthy controls using stable isotope technology. This study demonstrates for the first time significantly increased residence times of the most atherogenic lipoproteins IDL and LDL (despite normal levels of the latter) and might help explain the extremely high prevalence of cardiovascular disease in these patients.

Subjects and Methods

Study Design

The two kinetic studies followed corresponding protocols previously described[20–22] and approved by the Internal Review Boards of the Philipps University of Marburg, Germany, the Innsbruck Medical University, Austria, and the Jikei University School of Medicine, Tokyo, Japan. Informed, written consent was obtained from each study participant before the study. The study was performed in all HD patients 1 day after dialysis. For 3 days preceding the study, all study participants received a standardized isocaloric diet composed of 30 kcal/kg body weight, 50% carbohydrates, 30% fat, and 20% protein with a maximum of 300 mg cholesterol/d. After starting following a ten-hour overnight fasting period, all study participants maintained their fast during the first 12 hours of the study. Two plastic indwelling catheters were placed intravenously in contralateral arm veins: one catheter was used for the tracer infusion and the other was used for the frequent blood sampling during the study. Trideuterated L-leucine (99% pure; Cambridge Isotopes Laboratories Inc) was administered as a priming bolus of 1.34 and 1.00 mg/kg for Austrian and Japanese study subjects, respectively, followed immediately by a constant infusion of 22 (Austrian) or 17 (Japanese) μg/kg per min for up to 12 hours. Blood samples (9 mL) were drawn into tubes containing EDTA (1 g/L) before tracer injection 10, 20, 30, 40, 60, 90, and 120 minutes thereafter, then hourly for up to 12 hours and daily for up to 1 week. Plasma was obtained by low-speed centrifugation and kept on ice until use.

Study Participants

A total of 12 male end-stage renal disease (ESRD) patients treated with HD (7 Austrians and 5 Japanese) and 13 male healthy controls (9 Austrians and 4 Japanese, selected from university and hospital staff) were studied. The mean age of HD patients was 51±13 years, whereas the age of healthy control subjects was 35±10 years. Dialysis was performed 3 times weekly for 4 hours on average. Dialysis adequacy was monitored monthly by calculating Kt/V values. In case of falling below a value of 1.2, dialysis session length was increased accordingly resulting in a value of 1.3 in 10 and 1.2 in 2 patients. HD patients had been dialyzed for an average of 66 months before the study. None of the study subjects had diabetes mellitus or any history of cardiovascular disease, familial hyperlipidemia, and nephrotic syndrome affecting the lipid metabolism at the time of this study. HD patients took vitamins, erythropoietin, and bicarbonate but had never received any lipid-lowering medication. The primary reason for developing ESRD as well as further clinical characteristics of all subjects are summarized in Table 1.

Preparation of Lipoproteins and Apolipoproteins

VLDL (d<1.006 g/mL), IDL (d=1.006 to 1.019 g/mL), and LDL (d=1.019 to 1.063 g/mL) were isolated by sequential preparative ultracentrifugation from 5 mL of plasma (50.3 Ti rotor, L-70K centrifuge; Beckman Instruments). VLDL-apoB, IDL-apoB, and LDL-apoB were isolated by preparative 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions. IDL were not collected from the Austrian study group.

Determination of Isotopic Enrichment

Apolipoprotein bands were excised from gels, hydrolyzed in 6 mol/L HCl at 110°C for 24 hours under nitrogen and lyophilized. Free amino acids were purified from plasma or protein hydrolysates by cation exchange chromatography (AG-50W-X6; Bio-Rad Laboratories) and then derivatized to n-heptafluorobutyrylisobutyl esters and, in the Austrian study, analyzed by gas chromatography/triple-stage quadrupole mass spectrometry in the chemical ionization and selected ion-monitoring mode, as previously reported.[23] The ions monitored were 363.1 m/z (mass-to-charge ratio) for unlabeled L-leucine and 366.1 m/z for labeled [2H3] L-leucine as parent ions (first mass spectrometry [MS]) and 280.1 m/z for the daughter ions of both types of leucine (second MS). In the Japanese study, isotopic enrichment was analyzed by gas chromotography (GC)-MS on a 6890 gas chromatograph connected to a 5973 quadrupole mass spectrometer (Hewlett Packard). Tracer enrichment was calculated as the tracer-to-tracee ratio, which is equivalent to the specific activity in radiotracer studies.[24]

Kinetic Modeling

A multicompartmental model was built using an interactive computer program (SAAMII, version 1.1; SAAM Institute Inc)[25] to determine apoB kinetic parameters. A previously published compartmental model[26] was used as the template for this study. Briefly, the plasma amino acid pool (compartment 1) was used as a forcing function, followed by a delay compartment (compartment 2) for lipoprotein assembly and subsequent secretion of lipoproteins from the liver. A single compartment was allocated for VLDL (compartment 3), IDL (compartment 4), and LDL (compartment 5). In the Austrian study group, the IDL compartment was excluded attributable to the lack of IDL data.

Individual percentage changes in plasma concentrations of VLDL-, IDL- and LDL-apoB, triglycerides, total and HDL-cholesterol were within 5% throughout the study period (data not shown), indicating steady state conditions. Therefore, the FCR was assumed to be equal to the fractional synthetic rate. Residence time equals 1/FCR. Fractional standard deviations, which equal the coefficient of variation of the respective parameter, for apoB FCR provided reasonable levels; the mean fractional standard deviation was 11.4±6.5% for VLDL-apoB, 18.3±17.2% for IDL-apoB, and 7.4±5.2% for LDL-apoB.

Because plasma volume (PV) has been shown to be increased in HD patients,[27–29] we adjusted PV values by hematocrit (Hct) using a recently reported formula by Mitra et al[30] (PV=blood volume [BV]·[1 to 0.86·Hct]). The factor 0.86 corrects for the difference between Hct levels in the systemic circulation and whole-body Hct

TABLE 1. Clinical Characteristics of HD Patients and Control Subjects

Subjects	Age, y	BMI, kg/m^2	Hct	Prot, g/dl	Alb, g/dl	CRP, mg/l	ApoE	Chol, mg/dl	LDL-C, mg/dl	HDL-C, mg/dl	TG, mg/dl	ApoB, mg/dl	Cause ESRD	Durat HD, mo	Creat, mg/dl	Urea, mg/dl
Controls																
1	27	22.3	0.37	6.5	4.2	1.9	3/3	141	90	34	86	66				
2	32	24.2	0.45	7.4	4.5	4.0	3/3	299	240	41	91	194				
3	50	27.6	0.48	5.8	3.6	5.8	4/3	204	125	36	216	125				
4	28	21.0	0.42	6.4	3.9	2.1	3/3	99	52	31	79	43				
5	36	30.6	0.45	7.0	4.8	1.0	4/3	196	126	45	124	116				
6	43	25.4	0.46	6.3	4.0	2.0	3/3	170	117	42	57	98				
7	23	22.5	0.45	6.5	4.1	2.0	2/2	150	93	37	100	150				
8	42	24.6	0.49	7.0	4.0	0.1	3/3	154	92	52	50	97				
9	21	22.0	0.45	7.0	4.4	0.1	3/3	187	113	56	90	94				
10	48	23.3	0.44	7.2	4.1	0.3	3/3	183	109	51	117	83				
11	45	22.9	0.45	7.3	4.2	0.9	3/3	173	107	49	86	85				
12	40	20.9	0.44	7.4	4.5	0.1	3/3	160	97	45	87	75				
13	23	22.0	0.45	8.4	5.3	0.1	3/3	182	100	59	113	79				
Patients																
1	31	26.5	0.34	6.7	3.9	4.7	3/3	184	129	27	140	163	GP	75	13.4	193
2	60	21.0	0.34	6.6	3.7	8.0	3/3	194	136	45	63	102	RAS	27	9.0	63
3	33	20.6	0.33	6.7	4.2	2.0	3/2	126	72	29	127	100	PN	108	13.5	170
4	61	22.3	0.33	6.5	3.6	12.0	3/3	168	115	31	111	84	SK	44	13.0	175
5	35	23.5	0.39	6.6	3.9	5.0	4/3	174	117	25	160	97	IgA	5	9.3	146
6	67	18.4	0.30	6.4	3.6	3.3	3/3	262	191	38	166	154	SK	41	9.7	94
7	61	24.5	0.33	7.1	3.7	2.0	3/3	253	168	35	250	163	PN	34	12.4	160
8	60	23.9	0.28	5.6	3.2	4.5	3/3	145	88	38	94	77	CGN	108	8.1	59
9	66	21.4	0.25	6.0	3.2	6.0	3/3	138	75	37	131	80	CGN	3	9.0	55
10	50	23.5	0.29	6.4	3.9	3.0	3/3	151	94	28	142	90	CGN	144	11.3	63
11	45	23.0	0.30	6.4	3.6	2.4	3/3	158	106	28	120	82	CGN	228	13.1	56
12	46	24.6	0.33	6.3	3.9	8.0	4/3	171	118	25	144	114	PKD	228	18.9	89
Controls, Mean±SD	35±10	23.8±2.8	0.45±0.03	6.9±0.7	4.3±0.4	1.6±1.7		177±46	112±43	45±9	100±41	100±39				
Patients, Mean±SD	51±13	22.8±2.2	0.32±0.04	6.4±0.4	3.7±0.3	5.1±3.0		177±42	117±36	32±6	137±45	109±33		66±88	11.7±3.0	110±54
P Value	0.005	0.610	0.000	0.049	0.001	0.001		0.740	0.530	0.002	0.008	0.430				

GP indicates Goodpasture syndrome; RAS, renal arterial stenosis; IgA, IgA nephritis; SK, small kidney; PN, pyelonephritis; CGN, chronic glomerulonephritis; PKD, polycystic kidney disease; CRP, C-reactive protein; Alb, albumin; BMI, body mass index; Chol, cholesterol; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; TG, triglycerides; ESRD, end-stage renal disease; Creat, creatinine.

Controls: subjects 1 to 9 (whites), subjects 10 to13 (Japanese); Patients: subjects 1 to 7 (whites), subjects 8 to 12 (Japanese)

levels as well as trapped plasma (≈4%). BV is similar between HD patients and controls when normotensive patients are selected.[31,32] Because patients in the present study were all normotensive, they were considered to have no intravascular fluid expansion. We therefore used 7% as the percentage BV (% BV/body weight) in both HD patients and control subjects.[33] Taken together, production rate (PR, mg/kg per day) was calculated by the formula PR=FCR · apolipoprotein concentration$_{\text{in plasma}}$ · (1 to 0.86 · Hct) · 0.07.

Quantification of Lipids, Apolipoproteins, and Routine Laboratory Parameters

Triglycerides, total cholesterol, and HDL cholesterol were measured with commercially available kits from Roche Diagnostics GmbH. LDL cholesterol was calculated with the Friedewald formula. ApoB concentrations of plasma and fractions thereof were measured by ELISA.[34] An affinity-purified polyclonal antibody against apoB (produced in our laboratory by immunizing rabbits with purified apoB) was used for coating and the same antibody, labeled with horseradish peroxidase, for detection. A calibrated standard (Apoproteins Human ApoB; Technoclone) served as a secondary standard. ApoE phenotyping was performed on delipidated plasma by isoelectric focusing. Urea, creatinine, total protein, albumin, and C-reactive protein were determined using standard assays on a COBAS INTEGRA analyzer (Roche Diagnostics).

Statistical Analysis

Data are presented as mean±SD, except as noted. For statistical analysis we used the nonparametric Mann-Whitney U test to test for significant differences between groups. Nonparametric correlations were calculated according to Spearman test. Significance is defined as $P<0.05$.

Results

Clinical Characteristics of HD Patients and Controls

Lipids and lipoprotein profiles of the 12 HD patients and of 13 healthy controls are summarized in Table 1. Total cholesterol, LDL cholesterol, and apoB concentrations were almost identical in both groups. Triglycerides were significantly higher (137 mg/dL versus 100 mg/dL) and HDL cholesterol significantly lower (32 mg/dL versus 45 mg/dL) as compared with controls, which is in accordance with previous findings.[12]

Kinetics of ApoB-Containing Lipoproteins

Figure 1 illustrates the mean tracer/tracee curves of apoB from the Austrian and Japanese studies, respectively. In both study groups, VLDL curves did not differ between HD patients and controls (not shown). In contrast, the LDL tracer/tracee curve increased more slowly in HD patients than in control subjects. In the Japanese study, the tracer/tracee curve of IDL-apoB from HD patients increased slowly, reached a peak around 20 hours, and then slowly decreased. This was in contrast to the control IDL-apoB curve, which peaked around 10 hours and thus closely followed the VLDL apoB tracer/tracee curve, particularly toward the second half of the study period. In line with the results from the Austrian study, the LDL apoB tracer/tracee curve was also slower than the control's counterpart and kept rising during the full study period of 48 hours.

Despite the fact that total cholesterol, LDL cholesterol, and LDL apoB levels were almost identical in HD patients and controls, we found dramatic differences in the in vivo kinetic parameters of IDL- and LDL-apoB between both groups, whereas the kinetic parameters of VLDL were not significantly different (Table 2 and Figure 2). Compared with controls, IDL-apoB FCR was one-third as high (2.87±1.02 versus 8.89±4.94 pools/d, $P=0.014$) in HD patients, accompanied by a 24% decrease in PR (9.05±3.08 versus 11.84±4.42 mg/kg-day) which did not, however, reach statistical significance ($P=0.221$). This resulted in a 60% increase in IDL-apoB levels in HD patients (6.2±1.8 versus 3.9±2.7 mg/dL). LDL-apoB FCR was significantly decreased to 50% in HD patients as compared with controls (0.22±0.12 versus 0.46±0.20 pools/d), which corresponded to a severely prolonged residence time of 4.6 days in HD versus 2.2 days in healthy controls. Furthermore, the LDL-apoB PR was significantly decreased in HD as compared with controls (9.8±4.9 versus 18.4±13.3 mg/kg-day).

Discussion

The major outcome of this study is the elucidation of an impaired metabolism of atherogenic lipoproteins which might significantly contribute to the high rate of cardiovascular disease in HD. We studied for the first time the in vivo kinetics of the atherogenic lipoproteins VLDL, IDL, and LDL in HD patients using stable isotope-labeling technology. Interestingly, the FCRs of IDL and LDL apoB were severely decreased in HD patients as compared with controls, whereas the in vivo kinetics of VLDL did not change significantly.

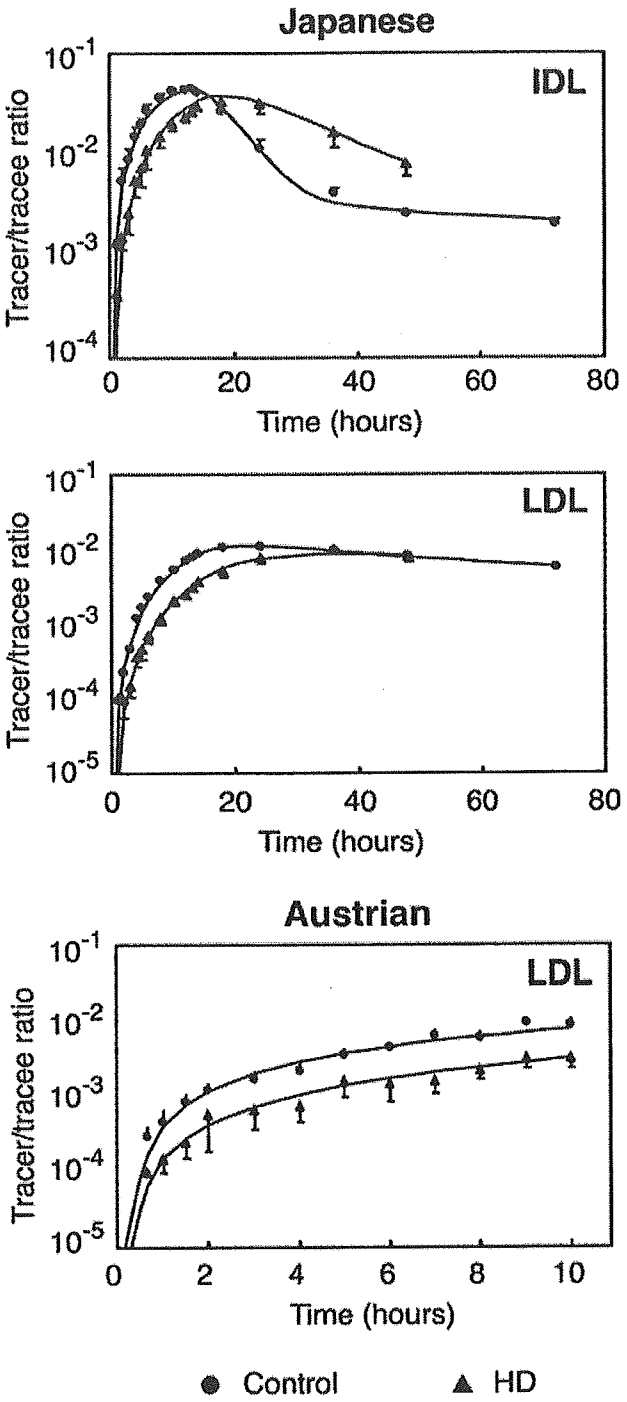

Figure 1. Tracer/tracee ratio curves for apoB-100 from IDL and LDL. ApoB tracer/tracee ratio curves of IDL and LDL were calculated from Japanese and Austrian HD patients (triangles) and controls (circles). Data were fitted by multicompartmental modeling using SAAMII. Bars represent standard error of means.

We did not investigate the metabolism of VLDL subfractions separately because it was previously shown in healthy subjects that FCR values of VLDL1 and VLDL2 did not differ from those of total VLDL.[35,36] We cannot, however, exclude that changes in the subfraction distribution in HD patients could be different compared with controls. Another limitation of the study is the assumption of comparable BVs