obesity-related metabolic syndrome and type 2 diabetes mellitus. Previous studies also showed that the serum A-FABP level predicts the development of metabolic syndrome [10] and was associated with carotid intima-media thickness [11], the number of stenotic coronary arteries [12], and coronary plaque volume determined by intravascular ultrasound (IVUS) [13]. These findings demonstrated the role of A-FABP as a potential mediator of atherosclerotic diseases.

Therefore, understanding the clinical significance of circulating levels of A-FABP may be useful for preventing the development of cardiovascular diseases. In this study, we investigated in a large population whether a higher plasma A-FABP level is significantly associated with the presence of CAD after adjustment for established cardiovascular risk factors. We also studied the relationship between A-FABP and CAD according to age.

Methods

Study group

This study included 211 consecutive patients (mean age: 66 years, range: 33-87 years) with coronary artery disease (CAD) recruited to undergo coronary angiography, from April 2008 to March 2009, at Kagawa Prefectural Central Hospital, Japan. Patients with CAD had 75% or greater organic stenosis of at least one major coronary artery, confirmed by coronary angiogram, had suffered a myocardial infarction, or had previously undergone percutaneous transluminal coronary angioplasty or coronary artery bypass graft surgery. Patients with hemodialysis, acute coronary syndrome, recent myocardial infarction within 4 weeks, and malignancies were excluded. Control male subjects (n = 211), matched with CAD patients for age (mean age: 66 years, range: 35-85 years), were selected from patients who visited our affiliated hospitals. Controls were characterized by no history of angina or other heart diseases, a normal resting ECG, and normal exercise ECG stress testing. This study protocol complied with the Declaration of Helsinki and was approved by the Ethics Committees of the institute. Informed consent was obtained from all patients before the study entry.

Clinical and Biochemical assessment

Blood samples were taken after overnight fasting. The plasma was separated and stored at-80°C, and plasma levels of A-FABP (Biovendor Laboratory Medicine, Modrice, Czech Republic) and high sensitivity C-reactive protein (hsCRP; R&D Systems, Minneapolis, MN) were measured by enzyme-linked immunosorbent assay, as previously described[13]. The performance characteristics of these assays were < 7 and < 8% CV intra-assay, and < 5 and < 7% CV inter-assay for A-FABP and hsCRP, respectively.

Risk factors were defined as follows. Diabetes was confirmed according to the criteria of the American Diabetes Association [14], or from a history of diabetes mellitus treatment. Dyslipidemia was defined as one or more of the following criteria: (1) serum triglyceride ≥150 mg/dL, (2) HDL-cholesterol < 40 mg/dL, (3) LDL-cholesterol ≥130 mg/dL, (4) already on lipid-lowering drugs. Hypertension was defined as a resting blood pressure of ≥140/90 mmHg or on regular antihypertensive medications. Smoking was defined as current smokers. eGFR was calculated by the Modification of Diet in Renal Disease (MDRD) equation [15] with coefficients modified for Japanese patients [16]: eGFR (ml/min/1.73 m^2) = 194 × (serum creatinine) $^{-1.094}$ × (age) $^{-0.287}$ *Ann Intern Med* **130** . Renal dysfunction was defined as eGFR < 60 ml/min/1.73 m^2 .

Coronary angiography

Coronary angiography was performed according to standard methods. After intracoronary injection of isosorbide dinitrate, angiograms were obtained in two or more views. The coronary angiogram was scored by two independent investigators and according to three techniques [17]: (1) *Vessel score:* The number of vessels with significant stenosis defined as 50% or greater luminal diameter narrowing. (2) *Stenosis score:* A modified Gensini score, which has been previously reported. Briefly, the most severe stenosis in each of eight segments was graded according to severity, from 1 to 4. The scores in each of the eight segments were added to give a total score out of 32. (3) *Extent score:* According to the proportional length of each vessel segment in the coronary artery tree, segments were graded with different maximum numbers of points, as previously reported. The scores of each vessel were added to give a total score out of 100. Stenosis score and extent score may be regarded to reflect the coronary plaque burden.

Statistical analysis

Continuous variables are presented as the mean ± SD or median (interquartile range) and differences between two groups were evaluated with an unpaired *t* -test or the Mann-Whitney U test, where appropriate. Categorical variables are presented by frequency counts, and intergroup comparisons were analyzed by the chi-square test. Data that were not normally distributed, determined using the Kolmogorov-Smirrnov test, were logarithmically transformed before linear regression analysis. Differences in plasma A-FABP across the scores of coronary angiography were compared by one-way analysis of variance (ANOVA), followed by the Bonferroni post-hoc test. Associations between CAD and all other parameters were first analyzed by simple logistic regression analysis and then by multivariate analysis. The multivariate

adjusted odds ratios (ORs) are presented with 95% confidence intervals (CIs). Statistical significance was defined as $p < 0.05$. Statistical analysis was performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL).

Results

Patient characteristics

Plasma A-FABP levels in CAD patients were significantly higher than in control subjects (median [IQR], 20.6 [15.7-27.8] vs. 15.1 [11.7-19.9], $p < 0.001$, Figure 1). The clinical characteristics of male CAD patients and control subjects are shown in Table 1. In the fourth quartile (plasma A-FABP level ≥24.5 ng/mL), the number of CAD patients was 2-fold that of control subjects, while in the first quartile, the number of CAD patients were less than that of control subjects. CAD patients had a significantly higher body mass index, fasting blood glucose, LDL cholesterol, and hsCRP, and lower level of HDL cholesterol and eGFR. The presence of hypertension, diabetes mellitus, dyslipidemia, and smoking habit was significantly higher in CAD patients than in control subjects.

Among diabetic patients (n = 155), the presence of CAD was higher in those with over the medium level of plasma A-FABP than in those with the lower plasma A-FABP (84% vs. 64%, $p < 0.01$). Among patients with dyslipidemia (n = 245), the presence of CAD was higher in those with over the medium level of plasma A-FABP than those with lower plasma A-FABP (78% vs. 54%, $p < 0.01$).

Table 1 Clinical characteristics of control subjects and CAD patients

	Control Subjects (n = 211)	CAD Patients (n = 211)
Age, years	66 ± 10	66 ± 11
Body mass index, kg/m^2	23.4 ± 2.9	24.9 ± 3.5*
Smoking, n(%)	29(13)	51(24)*
A-FABP (ng/mL)	15.1 (11.7-19.9)	20.6 (15.7-27.8)*
Diabetes Mellitus, n(%)	39(18)	116(55)*
FBS (mg/dL)	98(92-109)	104(95-126)*
Hypertension, n(%)	65(31)	156(72)*
Dyslipidemia, n(%)	83(39)	162(77)*
LDL-C (mg/dL)	105(85-122)	124(97-134)*
HDL-C (mg/dL)	58 ± 12	43 ± 11*
Triglycerides (mg/dL)	126 (105-151)	129(90-170)
eGFR(ml/min/1.73 m^2)	75.6 ± 17.6	66.9 ± 18.1*
hsCRP(mg/L)	0.67(0.29-1.68)	1.62(0.60-3.02)*
Medications		
ACEI/ARBs	28(13)	82(39)*
CCBs	44(21)	91(43)*
Diuretics	18(9)	30(14)
β-blockers	23(11)	54(26)*
Statins	55(26)	126(60)*
Thiazolidinediones	15(7)	23(11)

Data are the mean ± SD, median (interquartile range), or frequency counts, as appropriate. A-FABP, adipocyte fatty acid-binding protein; FBS, fasting blood glucose; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; hsCRP, high sensitivity C-reactive protein; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CCBs, calcium channel blockers.

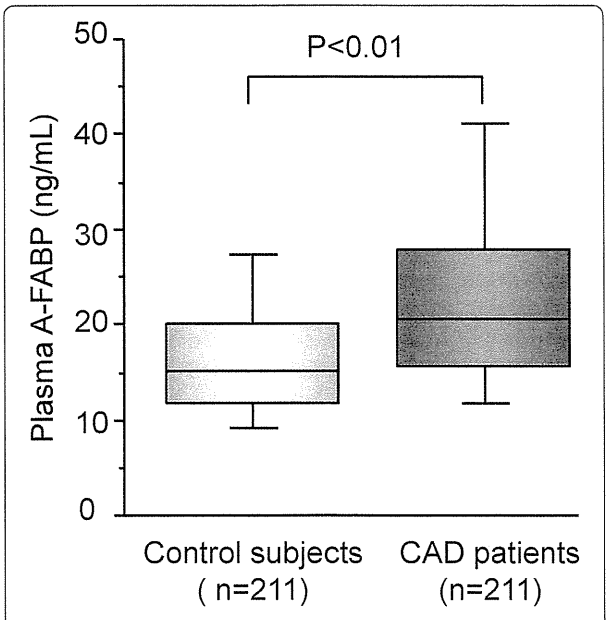

Figure 1 Box-and whisker plot showing plasma levels of A-FABP in CAD patients and control subjects. In these plots, lines within boxes represent median values; upper and lower lines in the boxes represent the 25th and 75th percentiles, respectively, and upper and lower lines outside boxes represent the 90th and 10th percentiles, respectively.

In all subjects, plasma A-FABP was correlated with the body mass index ($r = 0.47$, $p < 0.001$), but not with age ($r = 0.01$, $p = 0.86$), LDL-cholesterol level ($r = 0.07$, $p = 0.15$), or HbA1c ($r = 0.09$, $p = 0.15$). Plasma A-FABP in subjects with a history of smoking was significantly higher than that in subjects without a history of smoking (19.6 [15.1-27.2] vs. 17.2 [12.7-24.1], $p = 0.03$). In each CAD group or control group, medications did not affect the plasma A-FABP level (data not shown).

Plasma A-FABP and the severity of CAD

The association of plasma A-FABP and three angiographic scores, the vessel score, extent score, and modified Gensini score, were analyzed in CAD patients. The level of plasma A-FABP did not differ among patients with one diseased vessel (20.1 [15.3-28.1]), two diseased vessels (20.9 [15.8-27.4], and three diseased vessels (21.6 [16.7-20.9]). Meanwhile, the extent score and the modified Gensini score were correlated significantly with a stepwise increase in plasma A-FABP (Figure 2).

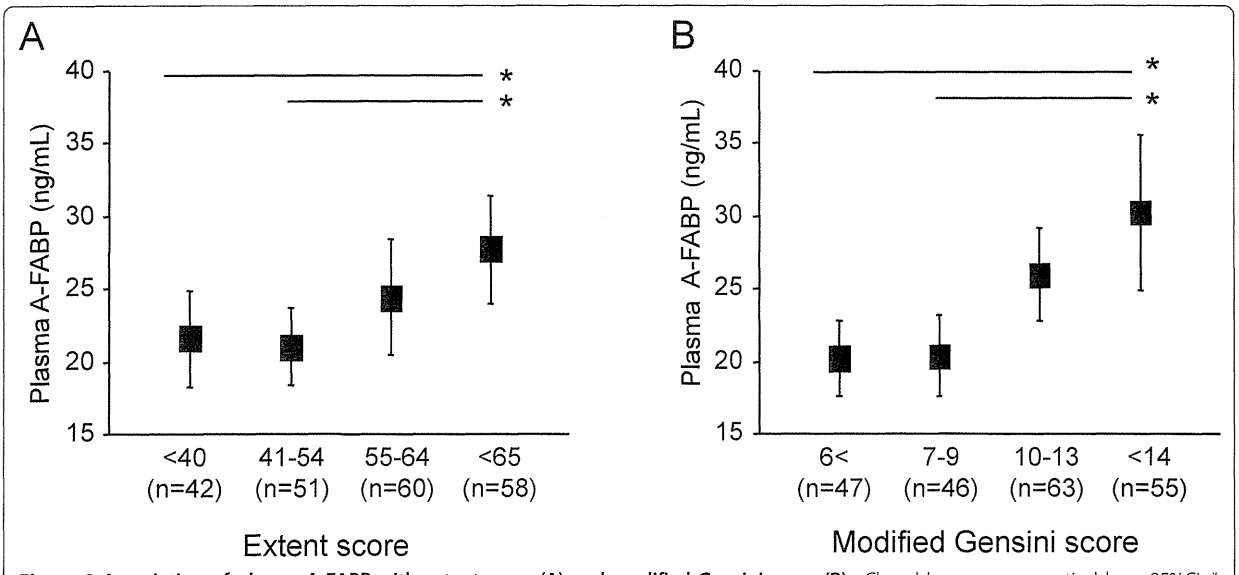

Figure 2 Association of plasma A-FABP with extent score (A) and modified Gensini score (B) . Closed boxes: mean; vertical bars: 95%CI. * $p < 0.05$

Plasma A-FABP and presence of CAD

To assess the relation of each factor with CAD patients, simple logistic regression analysis was performed (Table 2). The body mass index, smoking, plasma A-FABP level, diabetes mellitus, fasting blood glucose, hypertension, dyslipidemia, LDL cholesterol, HDL cholesterol, renal dysfunction, eGFR, and hsCRP were significantly associated with the presence of CAD. The uses of angiotensin-converting enzyme inhibitors/angiotensin II receptor blockers (ACEIs/ARBs), calcium channel blockers (CCBs), β-blockers, and statins were also significantly associated with the presence of CAD statistically; however, the following parameters were dependent on each other; fasting blood glucose and diabetes mellitus, lipid profile and dyslipidemia, and eGFR and renal dysfunction. Therefore, diabetes mellitus, dyslipidemia and renal dysfunction were selected as variates for multiple logistic regression analysis, along with body mass index, smoking, hsCRP, plasma A-FABP, and the use of ACEI/ARBs, CCBs, β-blockers, and statins. Multiple logistic regression analysis, including all the above factors revealed that the increase in plasma A-FABP was independently associated with the presence of CAD ($p = 0.01$) along with traditional CAD risk factors.

Furthermore, to evaluate the impact of plasma A-FABP on CAD presence according to age, we analyzed patients aged < 65 years and ≥65 years separately (Table 3). For analysis of subjects aged < 65, age-matched control subjects (n = 92) against CAD subjects aged < 65 years (n = 102) were re-selected from the control group. The mean ages of the control group and CAD group were comparable (57 ± 6 years and 57 ± 6 years, respectively). Simple logistic regression analysis demonstrated that plasma A-FABP, smoking, body mass index, diabetes mellitus, hypertension, dyslipidemia, renal dysfunction, hsCRP, and the use of ACEI/ARBs, CCBs, and statins were significant factors associated with the presence of CAD, Multiple logistic regression analysis including all significant factors in subjects aged < 65 years revealed that the increase in plasma A-FABP was independently associated with the presence of CAD ($p = 0.001$) along with smoking and diabetes mellitus. Next, for analysis of subjects aged ≥65, control subjects (n = 93) age-matched against CAD subjects aged ≥65 years (n = 109) were re-selected from the control group. The mean ages of the control and CAD groups were 75 ± 5 years and 75 ± 6 years, respectively. Simple logistic regression analysis demonstrated that plasma A-FABP, diabetes mellitus, hypertension, dyslipidemia, renal dysfunction, hsCRP, and the uses of ACEI/ARBs, CCBs, β-blockers and statins were significant factors associated with the presence of CAD; however, multiple logistic regression analysis including all significant variates revealed that plasma A-FABP was not an independent factor associated with CAD.

Discussion

In the present study, we demonstrated that higher level of plasma A-FABP in male subjects was independently associated with the presence of CAD after adjustment for established cardiovascular risk factors, such as smoking, diabetes mellitus, dyslipidemia, hypertension, and smoking. Our multivariate logistic analysis demonstrated that the adjusted odds ratio of plasma A-FABP (per doubling)

Table 2 Logistic regression analysis in whole subjects

	Simple		Multiple	
Factors	Crude OR(95%CI)	p	Adjusted OR (95%CI)	p
Body mass index (per kg/m^2)	1.163 (1.090-1.240)	< 0.001	1.024 (0.940-1.117)	0.584
Smoking (yes)	2.000 (1.201-3.308)	0.006	2.408 (1.140-2.703)	0.012
A-FABP (per doubling)	3.008 (2.158-4.194)	< 0.001	1.755 (1.140-2.703)	0.010
Diabetes Mellitus (yes)	5.385 (3.465-9.369)	< 0.001	4.896 (2.799-8.562)	0.001
FBS (per doubling)	11.978 (4.819-29.772)	< 0.001		
Hypertension (yes)	6.371 (4.169-9.737)	< 0.001	3.830 (1.755-8.360)	< 0.001
Dyslipidemia (yes)	5.099 (3.342-7.779)	< 0.001	1.857 (0.903-3.817)	0.092
LDL-C (per mg/dL)	0.992 (0.986-9.999)	0.024		
HDL-C (per mg/dL)	0.905 (0.887-0.823)	< 0.001		
Triglycerides (per doubling)	1.003 (1.000-1.007)	0.676		
Rrenal dysfunction (yes)	2.465 (1.559-3.899)	< 0.001	1.787 (0.946-3.375)	0.074
eGFR (per ml/min/1.73 m^2)	0.972 (0.961-0.984)	< 0.001		
hsCRP (per doubling)	1.379 (1.215-1.564)	< 0.001	1.436 (1.216-1.697)	< 0.001

Multiple logistic regression analysis included variates in bold in simple regression analysis and the use of ACEI/ARB, CCBs, β-blockers, and statins. R2 = 0.364 (n = 422)

for the presence of CAD was 1.78 (95%CI: 1.14-2.70, p = 0.01). Furthermore, sub-analysis based on age showed that this association remained significant in subjects aged < 65 years, but not in subjects aged ≥65 years.

A-FABP and CAD

Several lines of evidence have suggested the role of A-FABP in atherogenesis, and it is possible that the high plasma A-FABP levels in patients with CAD in this study were the cause of progressive coronary atherosclerosis. Studies on the molecular function of A-FABP in macrophages have shown that A-FABP deficiency reduced foam cell formation in response to oxidized LDL and increased the cholesterol efflux pathway[18]. A-FABP-deficient mice also showed a significant decrease of vascular atherosclerosis in the absence of differences in serum lipids or insulin sensitivity in hyperlipidemia model mice, and this effect was attributed to the action of A-FABP in macrophages[5]. Thus, A-FABP in macrophages and adipocytes has pathological effects on vessels. In humans, cohort studies suggested that A-FABP plays an important role in insulin resistance and metabolic syndrome [9]. Our study shows that diabetic patients over the medium level of plasma A-FABP had a significantly greater presence of CAD than diabetic patients with lower plasma A-FABP. These results suggest that plasma A-FABP may have a differential impact on CAD from diabetes mellitus. A previous cross-sectional study, however, showed that serum A-FABP was correlated closely with risk factors characterized by abdominal obesity, including insulin resistance, hyperglycemia, increased serum triglyceride, LDL-cholesterol, and decreased HDL-cholesterol[9]. Our findings also found that plasma A-FABP had a weak correlation with hsCRP and eGFR (data not shown). In addition, previous studies showed that medications affect the circulating A-FABP level. Serum A-FABP was reported to be reduced with statin therapy [19], but increased with the treatment of thiazolidinedione [20]. Despite several confounding factors, multiple logistic regression analysis demonstrated that plasma A-FABP was independently associated with the presence of CAD after adjustment for well-known CAD risk factors, hsCRP, and renal dysfunction. Thus, increased plasma A-FABP can be considered a candidate risk factor for CAD, although a large-scale prospective study in the general population is needed.

Table 3 Significant factors associated with CAD in younger and elder subjects

Subjects aged < 65 years	Adjusted OR (95%CI)	p
A-FABP (per doubling)	3.063 (1.343-6.987)	0.001
Smoking (yes)	2.877 (1.001-8.231)	0.04
Diabetes Mellitus (yes)	6.937 (2.759-17.438)	< 0.001
Subjects aged>65 years	Adjusted OR (95%CI)	p
Diabetes Mellitus (yes)	3.633 (1.602-8.238)	0.002
Hypertension (yes)	4.633 (1.602-8.238)	0.011
hsCRP (per doubling)	1.519 (1.210-1.908)	< 0.001

The model in subjects aged < 65 years included plasma A-FABP, smoking, body mass index, diabetes mellitus, hypertension, dyslipidemia, renal dysfunction, hsCRP, and the use of ACEI/ARBs, CCBs, and statins. The model in subjects aged>65 years included plasma A-FABP, diabetes mellitus, hypertension, dyslipidemia, renal dysfunction, and hsCRP, the use of ACEI/ARBs, CCBs, β-blockers and statins. Only statistical significant factors are presented in Table 3.

Impact of A-FABP on atherosclerosis

A-FABP is expressed in adipocytes and macrophages, and an increase in plasma A-FABP may reflect an increased expression in those cells. Plasma A-FABP concentration has been reported to be correlated with carotid atherosclerotic parameters, such as intima-media thickness[11]and plaque volume of the coronary artery determined with IVUS[13]. In line with previous studies, we demonstrated that plasma A-FABP in CAD patients increased with the severity of angiographic coronary stenosis[12]. Besides the severity of coronary stenosis, previous studies showed that plaque vulnerability is more important for future cardiac events[21]. Extent of atherosclerosis is another marker of an increased risk of coronary events. There is a significant association between coronary artery calcium scores, that reflects the actual presence and severity of atherosclerosis, and cardiovascular events [22]. In our study, A-FABP level was closely associated with modified Gensini score and extent score, that reflect coronary plaque burden. Thus, A-FABP might have potential to be a predictor of cardiovascular outcomes.

Several studies showed that genetic involvement in adipokine regulation was associated with atherosclerosis [23]. Regarding A-FABP, the T-87 C allele in the promoter region of the A-FABP gene has been shown to be associated with lower triglyceride levels, a reduced risk for coronary artery disease and type 2 diabetes [8]. Recent genetic studies identified several candidate genes associated with type 2 diabetes, including KCNQ1 [24], while A-FABP also contributed to the development of diabetes mellitus. In fact, a previous study showed that circulating A-FABP could predict the development of type 2 diabetes [10]. A basic experiment showed that interplay between FABPs and pancreatic islets plays an important role insulin secretion [25]. Thus, even though the direct interaction between A-FABP polymorphism and atherosclerosis has not been fully elucidated, the influence of A-FABP on lipid and glucose metabolism [26]may be one of the important mechanisms of atherosclerosis.

In this study, multivariate analysis using traditional CAD risk factors revealed that the association between elevated plasma A-FABP and the presence of CAD remain significant in patients aged < 65 years, but not in subjects aged ≥65 years. This finding is similar to predictive power of cardiovascular risk factors in relation to aging. Several studies documented that impact of risk factors on mortality decreases with age, partly because of selective survival and the influence of comorbidity on risk factor levels [27-29]. For instance, increased systolic blood pressure or cholesterol in younger subjects may affect cardiovascular mortality events with low-grade elevation or short-term exposure, more than in subjects of advanced age. On the other hand, the effect of each factor for risk control would be reduced in subjects of advanced age. As the progression of coronary artery disease is the manifestation of a cluster of several risk factors, the impact of A-FABP on the development of atherosclerosis would be reduced in subjects of advanced age; thus, the impact of increased plasma A-FABP can be regarded as a coronary risk factor especially in patients aged < 65 years.

Gender difference in circulating A-FABP

This study focused on men, because male gender is an important risk factor of CAD and gender differences in serum A-FABP have been reported [13]. Previously, we have shown that serum A-FAPB in female patients with CAD was significantly higher than in male patients with CAD, whereas serum A-FABP in men was lower than in women. This contradictory difference in serum A-FABP in women might confuse the analysis of the interaction; therefore, we analyzed only men in this study. This sex difference may be partly the result of the relatively higher fat percentage in women than in men, because adipose tissue is the major contributor of circulating A-FABP. Another explanation is the regulation of A-FABP expression by sex hormones. A study showed that the secretion of adiponectin, which is another major adipokine, was suppressed by testosterone [30]. A similar mechanism of the secretion of A-FABP might be possible. Further studies will be needed to elucidate the clinical implication of increased plasma A-FABP in women in respect of CAD.

Conclusion

An increase in circulating A-FABP in men was demonstrated to be independently associated with the presence of CAD after adjustment for established cardiovascular risk factors. Further, the plasma A-FABP had more impact on CAD in non-elderly men than in elderly men. Our findings indicated that A-FBAP might be a clinically significant mediator linking obesity and coronary atherosclerosis, and the measurement of circulating A-FABP may be useful to evaluate the risk of CAD.

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Authors' contributions
MD, TM, KT, MI, conceived the study, and participated in its design and coordination and helped to draft the manuscript.
SU carried out the immunoassays
SH, SK KN, KK, HI were involved in drafting the manuscript or revising it critically
All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.
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Prednisolone Ameliorates Idiopathic Pulmonary Arterial Hypertension

To the Editor:

Idiopathic pulmonary arterial hypertension (IPAH) is caused by pulmonary vascular remodeling. The entire mechanism by which pulmonary vascular remodeling develops has not been well elucidated. Inflammation or autoimmune background are implied in many cases (1, 2). However, there are no proven therapies that modulate inflammatory processes to treat IPAH. We have previously reported that prednisolone has an inhibitory effect on the proliferation and migration of pulmonary arterial smooth muscle cells (PASMC) from patients with IPAH (3). Here, we report a case of IPAH coexisting with idiopathic thrombocytopenic purpura (ITP), which was successfully treated with prednisolone. The severity of IPAH was ameliorated by prednisolone therapy.

A 34-year-old female, who was diagnosed with IPAH three years earlier, was admitted to our hospital for further evaluation and treatment of thrombocytopenia. After detailed examination by a hematologist, she was diagnosed with ITP. Bone marrow aspiration showed a normal number of megakaryocytes, and platelet-associated IgG was elevated to 135.4 ng/10^7 cells (normal range: 9.0–25.0 ng/10^7 cells). A rheumatologist excluded definite diagnosis of any collagen diseases. One mg/kg/day (65 mg/body/d) of oral prednisolone was started as a standard therapy for treatment of ITP (Figure 1). During prednisolone therapy, the dose of epoprostenol (94 ng/kg/min) and diuretics (trasemide, 8 mg/d) were maintained at the same levels. Her condition progressively had improved and she gradually lost 3.3 kg in body weight. She felt less dyspnea, and World Health Organization (WHO) functional class improved from class III to II. Six-minute-walk distance increased to 305 m, although she could not walk for 6 minutes before steroid therapy because of shortness of breath. The cardiothoracic ratio decreased from 64% to 56%. Pericardial effusion decreased from 17 to 4 mm and the Doppler-estimated peak systolic tricuspid regurgitation pressure gradient decreased from 75 to 50 mm Hg. No adverse effects were observed during prednisolone treatment. While tapering prednisolone, she started to feel slight increase in shortness of breath upon exertion. Platelet counts were successfully maintained within normal range after termination of steroid therapy. However, the tricuspid regurgitation pressure gradient again increased, and we resumed increasing the dose of epoprostenol. Although she was discharged from our hospital at that time, she underwent living-donor lobar lung transplantation due to progressive heart failure 5 months later.

It is well documented that inflammation is involved in the development of pulmonary vascular remodeling and the pathogenesis of IPAH. Elevated serum levels of interleukin-1β and -6 and circulating monocyte chemoattractant protein-1 are reported in patients (2, 4). T- and B-lymphocytes and macrophages are found to accumulate in plexiform lesions (1, 5). Prednisolone is a drug that has anti-inflammatory, immunosuppressive, and anti-proliferative effects (6, 7). The effectiveness of steroid therapy in pulmonary hypertension associated with collagen diseases is already reported (8–10). However, there is no report that demonstrates the effectiveness of prednisolone therapy in IPAH alone, without any association with collagen diseases.

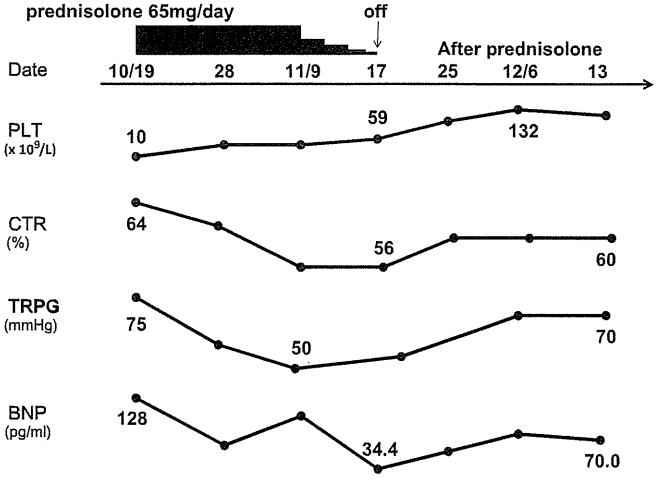

Figure 1. Time course of laboratory data and pulmonary hypertension before and after prednisolone treatment. Platelet counts (PLT), cardiothoracic ratio (CTR), Doppler-estimated peak systolic tricuspid regurgitation pressure gradient (TRPG), and plasma level of brain natriuretic peptide (BNP) are shown.

We have previously shown that prednisolone significantly inhibited platelet-derived growth factor (PDGF)-induced accelerated proliferation and migration of PASMC from patients with IPAH (3). Moreover, we demonstrated that the effect was caused, at least in part, by inhibiting activation of NF-κB, a key transcription factor that controls immunity, inflammation, cell proliferation, and apoptosis (11).

In summary, this case describes the potential effectiveness of prednisolone for the treatment of IPAH. The rationale for such treatment derives from its potent antiproliferative effect on PASMC from patients with IPAH demonstrated *in vitro*. We recognize that it is still difficult to integrate these results to apply prednisolone to all patients, taking the many side effects of prednisolone into account. However, this case supports the notion that prednisolone can inhibit pulmonary vascular remodeling and could be considered as a new targeted therapy for IPAH.

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An Unusual Localized Progressive Fibrotic Cavity Mimicking Lung Malignancy in Idiopathic Pulmonary Fibrosis

To The Editor:

Idiopathic pulmonary fibrosis (IPF) is defined as a chronic fibrosing interstitial pneumonia of unknown cause limited to the lungs (1). Because of the increased risk in developing lung cancer, physicians must make their best efforts in their differential diagnoses of pulmonary cavities in patients with IPF. In this report, we describe a cavitary lesion resembling a cavitary mass in IPF histopathologically diagnosed as localized progressive fibrosis.

A 72-year-old man was admitted for further evaluation of a cavitary lung mass. Three years previously, the patient had been diagnosed as having IPF (Figure 1A) and treated with oral prednisolone and cyclophosphamide. On high-resolution computed tomography (HRCT) at 4 months before admission, a cavitary nodule 2.3 × 1.5 cm in size and 5 mm in wall thickness in the left upper lobe had been initially detected (Figure 1B). HRCT on admission showed a cavity containing a mural nodule 3.7 × 1.9 cm in size and 13 mm in wall thickness (Figure 1C). Contrast-enhanced CT of chest revealed an enhancement of the cavitary wall with suspicion of an aspergilloma or pulmonary tuberculosis. However, there was no evidence of infection of bacteria or fungus in sputum and bronchial washing fluids. Percutaneous transthoracic needle biopsy was performed. Histopathologic examination disclosed chronic inflammation with dense fibrosis, focal organization, and infiltration of neutrophils. On follow-up HRCT at 10 months after discharge, the cavity regrew to 2.7 × 2.6 cm (Figure 1D). The wall thickness of the cavity was increased to 18 mm, with a volume decrease in the involved pulmonary lobe. Moreover, diffuse ground glass opacity with diffuse honeycombing predominantly in the subpleural location was aggravated as compared with previous scans. Fluoroscopically guided percutaneous lung biopsy was performed again, but there was no evidence for infection or malignancy. A wedge resection was performed for the cavitary mass. Pathologic examination showed nonspecific chronic inflammation with dense fibrosis, and negative results of fungal and mycobacterial stains (Figures 1E and 1F). There was no evidence for recurrence through 20 months after the resection.

This is an interesting case of a pulmonary cavitary lesion histopathologically diagnosed as progressive fibrosis by repetitive biopsies, which could be misdiagnosed as aspergilloma or lung malignancy on serial HRCT. IPF is associated with an increased risk of various pulmonary diseases such as lung cancer, pulmonary tuberculosis, aspergillosis, and other respiratory infectious diseases. In addition, our case supports the existence of a rare entity, the localized progressive fibrosis presenting as cavitary lesions in IPF, although the prevalence is very low. Therefore, a pulmonary cavity with preexisting IPF should always carefully be evaluated and confirmed even though an invasive diagnostic modality may be used. The localized progressive fibrosis in our patient suggests that pulmonologists may include it in their differential diagnoses of cavitary lung lesions developed in IPF.

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Is the Reference Arterial pH Higher than Usually Acknowledged?

To the Editor:

Since the invention of the blood gas apparatus by Severinghaus and Bradley in 1959 (1), arterial blood gas analysis has become

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CARDIOVASCULAR PATHOLOGY

Original Article

Elevated oxidative stress is associated with ventricular fibrillation episodes in patients with Brugada-type electrocardiogram without SCN5A mutation

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Abstract

Background: Brugada syndrome is a disease known to cause ventricular fibrillation with a structurally normal heart and is linked to SCN5A gene mutation. However, the mechanism by which ventricular fibrillation develops in cases of Brugada-type electrocardiogram without SCN5A mutation has remained unclear. Recently, oxidative stress has been implicated in the pathophysiology of cardiac arrhythmia. We also investigated oxidative stress levels in the myocardia of patients with Brugada-type electrocardiogram. **Methods:** Endomyocardial biopsy samples were obtained from 68 patients with Brugada-type electrocardiogram (66 males and two females). We performed histological and immunohistochemical analyses for CD45, CD68, and 4-hydroxy-2-nonenal-modified protein, which is a major lipid peroxidation product. **Results:** SCN5A mutation was detected in 14 patients. Ventricular fibrillation was documented in three patients with SCN5A mutation and in 11 without SCN5A mutation. In patients with SCN5A mutation, 4-hydroxy-2-nonenal-modified protein-positive area was not significantly different between the documented ventricular fibrillation (VF) group (VF+ group) and the group without documented VF (VF− group). However, in patients without SCN5A, the area was significantly larger in the VF+ group than that in the VF− group ($P<.05$). All other parameters (fibrosis area, CD45, and CD68) were not different between the VF+ and VF− group in both SCN5A+ and SCN5A− patients. **Conclusion:** Oxidative stress is elevated in the myocardium of patients with Brugada-type electrocardiogram who have VF episodes and do not have SCN5A gene mutations. Oxidative stress may be associated with the occurrence of VF in patients with Brugada-type electrocardiogram without SCN5A mutation.

Keywords: Oxidative stress; Ventricular fibrillation; Brugada syndrome

1. Introduction

Brugada syndrome (BS) is a disease characterized by ST-segment elevation in right precordial leads and episodes of ventricular fibrillation (VF) in the absence of structural heart disease [1]. About 20% of BS cases have been linked to mutations in the SCN5A gene, the gene encoding the alpha subunit of the cardiac sodium channel [2,3]. Functional analysis employing expression systems has revealed that mutations in SCN5A resulted in "loss of function" of I_{Na}, which reduces the inward sodium current, induces conduction delay, and predisposes the substrate for reentry. Other

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gene mutations such as CACNA1c [4], CACNB2b [4], GPD1-L [5], SCN1B [6], and KCNE3 [7] have also been reported. However, cases with such mutations are not frequent and the prevalence of those mutations is not clear [8]. Recently, Frustaci et al. [9] reported that lymphocytic myocarditis was observed in patients with Brugada-type electrocardiogram (ECG) who did not have SCN5A gene mutations, but the association with histological findings and occurrence of ventricular fibrillation (VF) has not been fully elucidated. Thus, the mechanism by which VF develops in cases of Brugada-type ECG without SCN5A mutation has remained unclear.

Recently, oxidative stress has been implicated in the pathophysiology of cardiac arrhythmia. Hydrogen peroxide (H_2O_2) decreases SCN5A transcription and current [10]. E2-isoketal, a highly reactive product of lipid peroxidation, potentiates inactivation of cardiac Na^+ channels [11]. Reactive oxygen species (ROS) contribute to cardiac sympathovagal imbalance in cardiomyocytes [12]. We also investigated oxidative stress levels, assessed by expression levels of 4-hydroxy-2-nonenal (HNE)-modified protein, a reliable marker of lipid peroxidation [13,14], in the myocardia of patients with Brugada-type ECG and investigated the association between VF events and oxidative stress levels in the myocardia of patients with Brugada-type ECG with and without mutation in the SCN5A gene.

2. Methods

2.1. Subjects

In the period from June 1998 to June 2008, we performed electrophysiological study and endomyocardial biopsy in 68 consecutive patients with Brugada-type electrocardiogram (ECG) (66 males and two females; mean age, 49.0 years). Brugada-type ECG was defined as coved ST-segment elevation (>0.2 mV) followed by a negative T-wave in more than one right precordial lead (V1 to V3) or third intercostal leads (V1 to V2) in the presence or absence of a sodium channel blocker (Fig. 1). Routine examinations, including cardiac echocardiography, coronary angiography, and right and left ventriculography, showed no evidence of structural heart disease in any of the patients. We examined the clinical characteristics of patients, including age, sex, spontaneous VF occurrence, history of syncope, family history of sudden death, and SCN5A mutation.

2.2. Cardiac catheterization, endomyocardial biopsy, and electrophysiological study

After providing written informed consent, all patients underwent cardiac catheterization, coronary angiography, right and left ventricular angiography, and endomyocardial

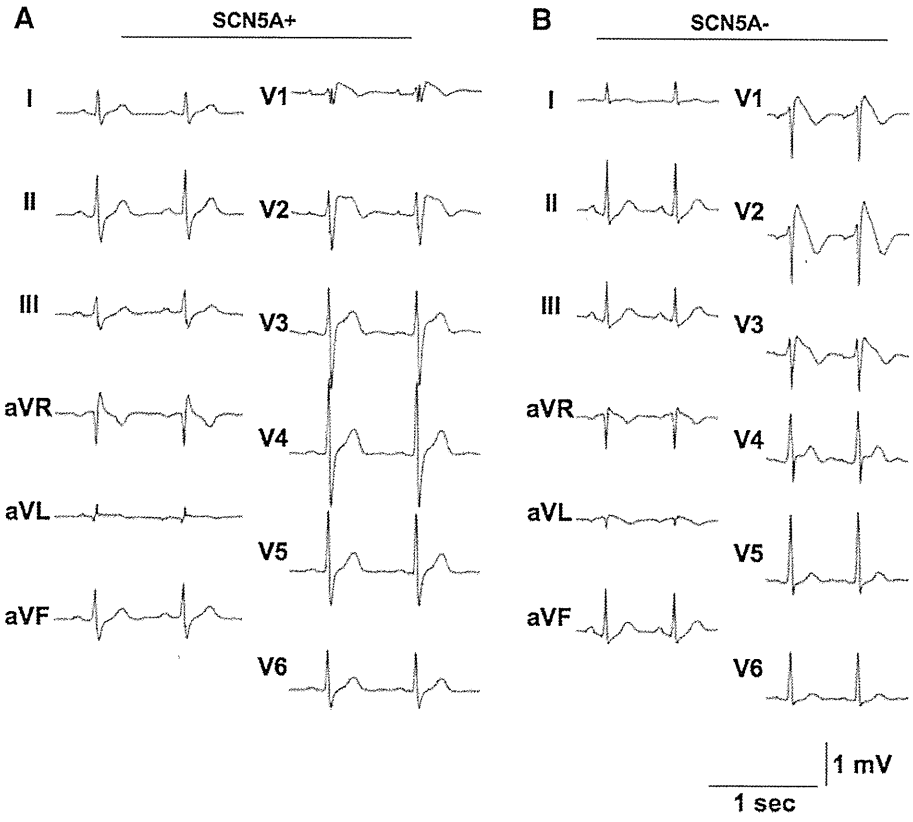

Fig. 1. Representative ECGs of patients with or without SCN5A mutation. (A) ECG of a patient with SCN5A mutation (SCN5A+), R282H (47 years old, male). (B) ECG of a patient without SCN5A mutation (SCN5A−) (42 years old, male).

biopsy. Endomyocardial biopsy samples (three or four per patient for histology) were obtained from the right ventricular (RV) side of the septum of all patients by the internal jugular approach.

The electrophysiological study was performed in all patients as reported previously [15,16]. The risks of the electrophysiological study were explained to each patient, and written informed consent was obtained from all patients. Induction of ventricular arrhythmia was initially attempted without the use of any antiarrhythmic drugs. The criterion for the induction of ventricular arrhythmia was induction of VF by programmed electrical stimulation from the RV apex, RV outflow tract, or left ventricle with a maximum of two extrastimuli at two cycle lengths.

2.3. Histology and immunohistochemistry

Endomyocardial biopsy samples were fixed in 10% formalin and embedded in paraffin. For histology, 5-μm-thick sections were cut and stained with hematoxylin and eosin and Masson's trichrome stain and examined by light microscopy.

Immunoenzymatic staining was performed using a DAKO LSAB System (Dako) according to the manufacturer's instructions, as previously described [13,14,17]. Briefly, the heart sections embedded in paraffin were preincubated with 1.5% hydrogen peroxide and normal BSA to block nonspecific reactions. CD45RO (1:100) and CD68 (1:50) antibodies (both from Dako) for the characterization of inflammatory infiltrate, and mouse monoclonal anti-HNE-modified protein antibody (1:50 dilution, NOF Medical Department) for assessment of oxidative stress were added. After incubation at 4°C overnight, the sections were incubated with biotinylated anti-mouse immunoglobulin for 20 min and subsequently with horseradish peroxidase-labeled streptavidin solution for 20 min. The slides were rinsed in cold Tris-buffered saline after each step of incubation. Peroxidase activity was visualized with diaminobenzidine (DAB) tetrahydrochloride solution.

2.4. Semiquantitative analysis of stained samples

Digital images of stained sections were taken with a Fujix Digital Camera HC-300/OL mounted on an Olympus BH-2 microscope. Color images from five randomly selected separate high-power fields (×200) in three or four sections per patient were obtained. Staining was analyzed using WinROOF Image software (Mitani Corp.) and assessed by using the following equation: stained area (%)=100×stained area (cm^2)/total sample size (cm^2).

CD45RO- and CD68-positive cells were counted by the following equation: inflammatory cell infiltration=number of CD45RO- or CD68-positive cells (n)/total sample size (cm^2).

2.5. Genetic analysis

Genetic analysis was performed in compliance with the guidelines for human genome studies of the Ethics Committee of Okayama University. Informed consent was obtained from all subjects. Genomic DNA was extracted from peripheral blood leukocytes by using a DNA extraction kit (Gentra, Minneapolis, MN, USA) and was stored at −30°C until use.

Twenty-seven exons of the SCN5A gene were amplified with previously reported intronic primers [18]. SCN5A gene exon 1 is a noncoding region, and we did not analyze this region in this study. Exons 6, 17–1 sense, 21, and 25 were not able to be sufficiently amplified by the primers, and we therefore designed the following intronic primers as previously described [19,20]. The primers used in this study are as follows: 5′-GTT ATC CCA GGT AAG ATG CCC-3′ (sense) and 5′-TGG TGA CAG GCA CAT TCG AAG-3′ (anti-sense) for exon 6; 5′-AAG CCT CGG AGC TGT TTG TCA CA-3′ (sense) for exon 17–1; 5′-TGC CTG GTG CAG GGT GGA AT-3′ (sense) and 5′-ACT CAG ACT TAC GTC CTC CTT C-3′ (anti-sense) for exon 21; 5′-TCT TTC CCA CAG AAT GGA CAC C-3′ (sense) and 5′-AAG GTG AGA TGG GAC CTG GAG-3′ (anti-sense) for exon 25. PCR was performed in a 20-μl reaction volume containing 50 ng of genomic DNA, 20 pmol of each primer, 0.8 mM dNTPs, 1× reaction buffer, 1.5 mM $MgCl_2$, and 0.7 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) or TAKARA Taq (Takara Bio, Inc., Otsu, Shiga, Japan). All PCR products were treated with exonuclease I (New England BioLabs, Ipswich, MA, USA) and shrimp alkaline phosphatase (USB Corporation, Cleveland, OH, USA), reacted with a Big Dye Terminator v. 1.1 cycle sequencing kit (Applied Biosystems) and analyzed on an ABI PRISM3130 XL sequencer (Applied Biosystems). The mutations were confirmed four times by independent PCR amplification and sequencing.

2.6. Statistical analysis

Data are all expressed as means±S.D. Intergroup comparison was done by Fisher's Exact Probability Test, and difference in mean values was tested by Student's t test,

Table 1
Patients' characteristics

Number	68
Age, years	49.0±11.6
Male/female	66/2
Family history of SCD (%)	19 (16.1)
Syncope (%)	12 (27.9)
ICD Implantation (%)	22 (32.8)
SCN5A Mutation (%)	14 (20.6)
Documented VF (%)	14 (20.6)
SCN5A mutation+ (%)	3 (4.4)
SCN5A mutation− (%)	11 (16.2)

Data are mean±S.D.
SCD: Sudden cardiac death; ICD: implanted cardioverter defibrillator; VF: ventricular fibrillation.

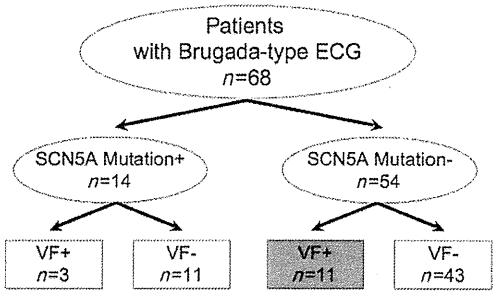

Fig. 2. Study profile.

at a critical level of 5% or lower. All data were analyzed using SPSS software (version 11.0.1).

3. Results

3.1. Patients' characteristics

Clinical characteristics of all patients with Brugada-type ECG are shown in Table 1. SCN5A mutation was detected in 14 patients. VF was documented in three patients with SCN5A mutation and in 11 patients without SCN5A mutation (Table 1 and Fig. 2).

Eleven patients (two patients with SCN5A mutation and nine patients without mutation) had histories of spontaneous VF that was converted to sinus rhythm by an external defibrillator before admission. In the other three patients (one patient with SCN5A mutation and two patients without the mutation), spontaneous VF occurred de novo after discharge from our hospital and was terminated by implantable cardioverter defibrillator therapy. There was no death in any of the patients.

3.2. Histology and immunohistochemistry

HNE-modified protein-positive area in patients with documented VF (VF+ group) was larger than that in patients without documented VF (VF− group) (VF+ group: 16.3 ±10.5% vs. VF− group: 9.3±5.7%, $P<.05$) (Fig. 3A). There were no significant differences in area of fibrosis and number of CD45RO- and CD68-positive cells between the VF+ and VF− groups.

We also checked those parameters in patients with and without SCN5A mutation. HNE-modified protein-positive areas were not significantly different in the SCN5A+ and SCN5A− patients (SCN5A+ group: 13.3±7.6% vs. SCN5A − group: 10.1±7.3%, P=NS). In SCN5A+ patients, HNE-modified protein-positive area was not significantly different between the VF+ and VF− group (VF+ group: 14.0±8.8% vs. VF− group: 12.7±7.5%, P=NS) (Fig. 3B). However, in patients without SCN5A (SCN5A−), the area was significantly larger in the VF+ group than that in the VF−

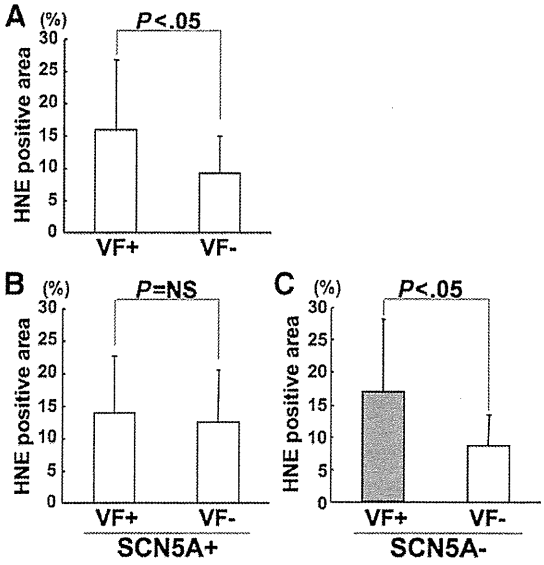

Fig. 3. HNE-modified protein-positive area. (A) HNE-modified protein-positive area in patients with spontaneous VF (VF+ group) vs. without (VF− group). (B) HNE-modified protein-positive area in patients with SCN5A mutation (SCN5A+). (C) HNE-modified protein-positive area in patients without SCN5A mutation (SCN5A−). Data are expressed as means±S.D.

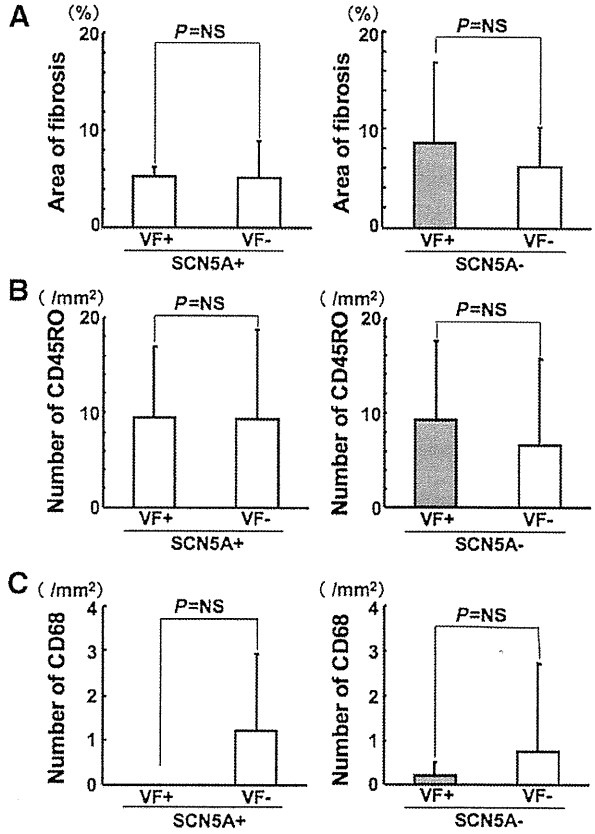

Fig. 4. Histological and immunohistochemical analyses. (A) Areas of fibrosis in SCN5A+ and SCN5A− patients in the VF+ and VF− groups. (B) Numbers of CD45RO-positive cells in SCN5A+ and SCN5A− patients in the VF+ and VF− groups. (C) Numbers of CD68-positive cells. Data are expressed as means±S.D.

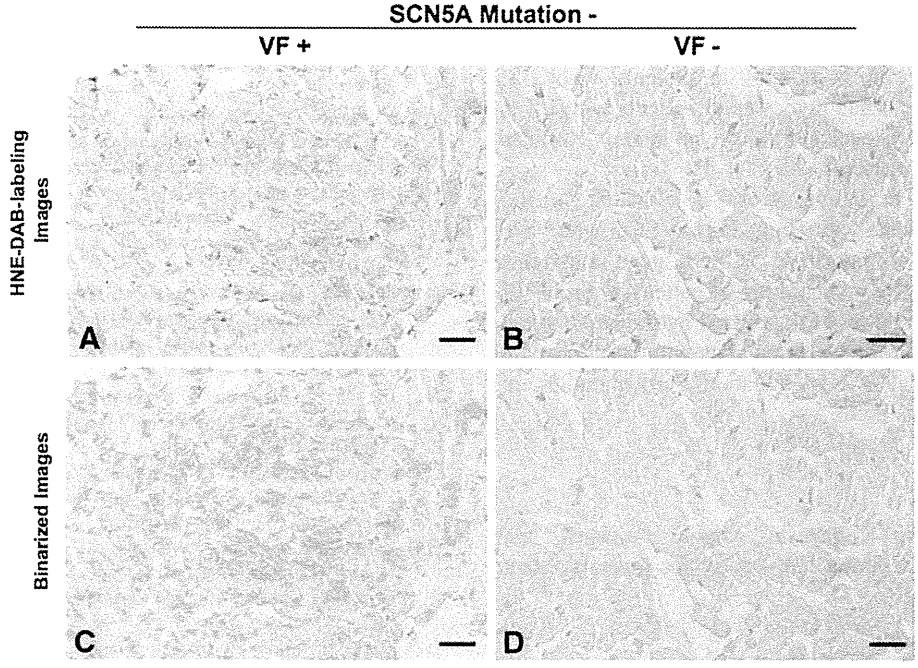

Fig. 5. Representative figures of HNE-modified protein staining. Representative immunostainings (brown) for HNE-modified protein by diaminobenzidine (DAB) (A and B) and binarized images (green) using WinROOF Image software (C and D) in the myocardium from a patient without SCN5A mutation and with spontaneous VF (A and C) and from a patient without SCN5A mutation and without spontaneous VF (B and D).

group (VF+ group: 17.0±11.2% vs. VF− group: 8.4±4.9%, $P<.05$) (Fig. 3C).

Area of fibrosis was not different between the VF+ and VF− groups in both SCN5A+ and SCN5A− patients (Fig. 4A). The number of CD45RO-positive cells was not significantly different between the VF+ and VF− groups in both SCN5A+ and SCN5A− patients (Fig. 4B). Infiltration of CD68-positive cell was rarely seen in patients in both the VF+ and VF− groups with or without SCN5A mutation (Fig. 4C).

Fig. 5 shows representative immunostainings (A and B) for HNE-modified protein in the myocardium from a patient without SCN5A mutation and with spontaneous VF (A and C) and from a patient without SCN5A mutation and without spontaneous VF (B and D). Positive immunostainings (brown) for HNE-modified protein are distinct in the cytosol of cardiac myocytes from a patient with spontaneous VF (Fig. 5A).

4. Discussion

We investigated oxidative stress levels in the myocardia of patients with Brugada-type ECG and also examined the relationship between oxidative stress levels and VF episodes. The major new finding of this work is that oxidative stress is elevated in the myocardium of patients with Brugada-type ECG who have VF episodes and do not have SCN5A gene mutations. Oxidative stress may play an important role in the occurrence of VF in patients with Brugada-type ECG without SCN5A gene mutations.

Oxidative stress induces loss of function of I_{Na}. Shang et al. [10] reported that H_2O_2 decreases SCN5A mRNA transcription and I_{Na} current. Fukuda et al. [11] reported that E2-isoketal, a highly reactive product of lipid peroxidation, potentiates inactivation of cardiac Na^+ channels. Our data showed that oxidative stress was elevated in the myocardium of BS patients with VF episodes who do not have SCN5A gene mutations. These findings indicated that loss of function of I_{Na} caused by oxidative stress is associated with the occurrence of VF in patients with Brugada-type ECG.

Oxidative stress is not related to the occurrence of VF in patients with Brugada-type ECG who have SCN5A mutation in our study. Frustaci et al. [9] reported that carriers of SCN5A mutations demonstrate myocardial cell degeneration and death. Therefore, mechanisms of VF occurrence in Brugada-type ECG patients with SCN5A mutation may be different from those in patients without SCN5A mutation. Further studies are needed to clarify the mechanisms. Since HNE-modified protein-positive areas were not significantly different in the SCN5A+ and SCN5A− patients in our study, it was thought that loss of function of the sodium channel due to SCN5A mutation did not cause oxidative stress.

ROS cause damage to lipid cell membranes in the process of lipid peroxidation. In this process, several aldehydes, including HNE, are generated as final products. HNE is recognized as the most reliable marker of lipid peroxidation [13,14]. Furthermore, exposure to a large amount of HNE (400 μmol/l) increases rat cardiac Na^+ current and causes cytotoxic effects in cardiac myocytes [21,22]. However, a small amount of HNE does not have any detectable gating

effects on I_{Na}, including I_{Na} decay, voltage-dependent activation, or the voltage dependence of channel availability [11]. Cardiac function is normal in patients with BS. Therefore, HNE in cardiac myocytes in patients with BS is thought to be at a low level and to have no cytotoxic effects and/or effect on I_{Na} current.

In conclusion, oxidative stress is elevated in the myocardium of patients with Brugada-type ECG who have VF episodes and do not have SCN5A gene mutations compared to that in the myocardium of patients without VF episodes. Loss of function of I_{Na} caused by oxidative stress may be a mechanism for VF in patients with Brugada-type ECG who do not have SCN5A mutation. Further studies are needed to clarify this point.

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Ezetimibe improves postprandial hyperlipemia and its induced endothelial dysfunction

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ABSTRACT

Objective: Postprandial hyperlipemia has been shown to impair endothelial function and contribute to the development of atherosclerosis. We investigated the association between postprandial lipid profiles and endothelial function, and we examined the effects of ezetimibe on postprandial hyperlipemia and lipemia-induced endothelial dysfunction.
Methods: A randomized prospective trial in which 10 mg/day of ezetimibe was administered to 10 subjects for 4 weeks and not administered to 10 subjects (control group) was performed. Lipid profiles and endothelial function, assessed by brachial artery flow-mediated dilation (FMD) during a fasting state and at 2, 4, 6 and 8 h after an oral cookie loading test, were determined before and after treatment for 4 weeks.
Results: In all subjects before treatment, the maximum reduction in postprandial %FMD was significantly correlated with the maximum increases in postprandial triglyceride (TG) ($r=-0.499$, $P<0.05$) and apolipoprotein B-48 (apoB-48) concentrations ($r=-0.551$, $P<0.05$). Ezetimibe treatment for 4 weeks significantly suppressed postprandial elevation in TG (area under the incremental curve, from 1419 ± 594 to 968 ± 321 mg h/dl, $P<0.05$), remnant lipoprotein cholesterol (from 66.9 ± 27.6 to 38.9 ± 15.4 mg h/dl, $P<0.01$) and apoB-48 (from 58.8 ± 27.5 to 36.2 ± 17.0 μg h/ml, $P<0.05$) concentrations, and postprandial endothelial dysfunction assessed by %FMD (maximum reduction in %FMD, from $-2.6\pm1.1\%$ to $-1.2\pm0.8\%$, $P<0.05$), whereas no significant changes were observed in the control group.
Conclusion: Postprandial hyperlipemia is closely correlated with transient endothelial dysfunction. Ezetimibe improves postprandial hyperlipemia and its induced endothelial dysfunction.

1. Introduction

A large number of studies have demonstrated that postprandial hyperlipemia contributes to the development of atherosclerosis and coronary heart disease (CHD) [1–3]. Postprandial lipemia is a physiological phenomenon occurring several times a day after ingestion of dietary fat when the dietary sources of fat exceeded the actual needs. Disturbances of triglyceride (TG) metabolism also induce prolonged postprandial hyperlipemia. Patients with obesity, diabetes and metabolic syndrome often have postprandial hyperlipemia [4] and exaggerated postprandial hyperlipemia has been observed even in fasting normolipidemic subjects [5]. In such circumstances, TG-rich lipoproteins (TRLs), which consist of chylomicrons assembled by TG, dietary cholesterol and apolipoprotein B-48 (apoB-48), have been shown to induce endothelial dysfunction, an initial process of atherogenesis, through enhanced inflammation and oxidative stress. Norata et al. showed that postprandial TRLs upregulate the expression of pro-inflammatory cytokines, inducing the impairment of brachial artery endothelial function beyond 8 h postprandially in both hyperlipidemic and normolipidemic subjects [6]. Moreover, van Oostrom et al. demonstrated that postprandial lipemia contributes to the recruitment of neutrophils with concomitant production of pro-inflammatory cytokines and oxidative stress, resulting in endothelial dysfunction in healthy normolipidemic subjects [7]. Thus, even in healthy volunteers, postprandial lipemia has been associated with the activation of leukocytes and upregulation of pro-inflammatory cytokines on the endothelium. Actually, several studies have demonstrated that postprandial hyperlipemia caused by oral fat intake induces impairment of flow-mediated dilations (FMD) of the brachial artery in healthy volunteers [8,9].

Ezetimibe, a novel lipid-lowering drug that selectively inhibits cholesterol absorption by inhibiting Niemann-Pick C1 like 1 (NPC1L1) protein, is commonly used for treatment of dyslipidemia. Recently, a clinical trial by Masuda et al. reported that ezetimibe improves fasting and postprandial hyperlipemia by suppression of

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intestinal chylomicron production in patients with type IIb hyperlipemia [10]. However, the effect of ezetimibe monotherapy on postprandial hyperlipemia-induced endothelial dysfunction was not fully evaluated.

Accordingly, the aim of the present study was to determine the association between postprandial lipid profiles and endothelial function, and to determine the effects of ezetimibe monotherapy on postprandial hyperlipemia and lipemia-induced endothelial dysfunction.

2. Methods

2.1. Study populations

Twenty volunteers, including 17 men and 3 women, were enrolled in this study. The inclusion criteria were aged of 20 years or above and not receiving lipid-lowering medications. Subjects were excluded from the study if they had undergone major surgery within 3 months prior to enrollment or if they had concomitant inflammatory diseases or malignant tumors. A randomized prospective trial in which ezetimibe (10 mg/day) was administered to 10 subjects for 4 weeks (ezetimibe group) and not administered to 10 subjects (control group) was performed. Lipid profiles and endothelial function, assessed by brachial artery FMD during a fasting state and at 2, 4, 6 and 8 h after an oral cookie loading test, were determined before and after treatment for 4 weeks. None of the 20 subjects had hypertension, diabetes, or cerebrovascular or cardiovascular disease, but 9 subjects met the diagnostic criteria for dyslipidemia, one subject met the criteria for metabolic syndrome, and 11 subjects were previous or current smokers. None of the subjects had received any medications. Hypertension was diagnosed according to the 1999 World Health Organization-International Society of Hypertension Guideline [11]. Diabetes was defined as a fasting blood glucose level ≥126 mg/dl and hemoglobin A1c (HbA1c) ≥6.1%. Dyslipidemia was diagnosed according to the 2007 Japan Atherosclerotic Society Guideline [12]. Metabolic syndrome was defined as waist circumference ≥85 cm for men or ≥90 cm for women as an essential component combined with 2 or more of the following components according to the 2005 definition and diagnostic criteria of metabolic syndrome in Japanese: TG ≥150 mg/dl and/or high-density lipoprotein cholesterol (HDL-C) <40 mg/dl; systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg; fasting blood glucose ≥110 mg/dl.

All of the studies were approved by the Ethics Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, and written informed consent was obtained from all subjects before the procedure.

2.2. Study protocol

2.2.1. Oral fat load (cookie test)

For fat loading, a cookie test was performed after overnight fasting for at least 8–12 h. The cookie consisted of 75 g carbohydrate (flour starch and maltose), 28.5 g fat (butter) and 8 g protein for a total of 592 kcal a carton (SARAYA Corp, Osaka, Japan) [13,14]. Subjects are given 30 g fat/m^2 body surface area, and they are instructed to ingest the cookie with water within 20 min. Time measurement was started when half of the cookie had been ingested. Venous blood samples were drawn and endothelium-dependent vascular function, assessed by FMD of the brachial artery, was determined during the fasting state before cookie ingestion and at 2, 4, 6 and 8 h after the cookie load. Endothelium-independent dilation, assessed by nitroglycerin-mediated dilation (NMD), was also measured during the fasting state before cookie ingestion and at 4 and 8 h after the cookie load. Measurements of FMD and NMD were performed by the same skillful technician who was blinded to the study design and medication status.

2.2.2. Biochemical analysis

The following parameters during the fasting state before cookie ingestion were measured: serum total cholesterol (Total-C), TG, low-density lipoprotein cholesterol (LDL-C), HDL-C, malondialdehyde-modified (MDA)-LDL cholesterol, remnant lipoprotein cholesterol (RLP-C), apoB-48, adiponectin, monocyte chemotactic protein-1 (MCP-1) and plasma glucose levels. HbA1c levels were measured using a high-performance liquid chromatography (HPLC) method. Concentrations of fasting plasma insulin were measured using a chemiluminescent enzyme immunoassay (CLEIA) method. Lipid profiles and other markers were measured at SRL Co., Ltd., Tokyo, Japan. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as [fasting plasma glucose (mg/dl) × fasting plasma insulin (μIU/ml)/405] and glucose intolerance was defined as HOMA-IR ≥2.0. Serum Total-C, TG, LDL-C, MDA-LDL, RLP-C, apoB-48 and plasma glucose levels were measured at 2, 4, 6 and 8 h after the cookie load. To compare the postprandial changes before and after treatment for 4 weeks in these parameters, area under the curve (AUC) was calculated using the trapezoidal method.

2.3. Assessment of endothelial vasomotor function

Endothelium-dependent and -independent dilations were assessed as parameters of vasodilation according to the guidelines for ultrasound assessment of FMD of the brachial artery [15]. Using a 10-MHz linear array transducer probe (Unex Co. Ltd., Nagoya, Japan), longitudinal images of the brachial artery at baseline were recorded with a stereotactic arm, which was used for optimal transducer positioning on the brachial artery proximal to the bifurcation of the radial and ulnar arteries, and measurements of brachial artery diameter were made after supine rest for at least 5 min. The diameter of the artery was measured from clear anterior (media–adventitia) and posterior (intima–media) interfaces, which were manually determined. Then suprasystolic compression (50 mmHg above systolic blood pressure) was performed at the right forearm for 5 min, and measurements of brachial artery diameter were made continuously from 30 s before to at least 2 min after cuff release. After at least 10 min of rest from FMD measurement, brachial artery diameter at baseline and for 5 min after administration of sublingual nitroglycerin 0.3 mg was also measured. Maximum vasodilation was then evaluated from the change in artery diameter after release of occlusion (%FMD) and after administration of nitroglycerin (%NMD).

2.4. Statistical analysis

Sample size was determined on the basis of the estimated FMD reported in other recent studies [16]. We assumed that the mean improvement in postprandial %FMD was 2.7% and the standard deviation (SD) was 2.0%. For using a two-sided test for differences, a minimal sample size of 10 patients was required in each group to detect statistical differences in %FMD with a power of 80% and an α-type error of 5% in statistical analysis. Results are expressed as means ± SD and data in the figure are presented as means ± standard error (SE). Categorical variables were compared using the chi-square test or Fisher's exact test. The two groups were compared using the Mann–Whitney *U*-test. Differences in lipid profile and endothelial function before and after 4 weeks in the two groups were compared using the Wilcoxon signed-ranks test. Pearson correlation coefficients were used to assess the relationships between maximum reduction in postpran-

Table 1
Baseline clinical characteristics.

	Total ($n=20$)	Ezetimibe ($n=10$)	Control ($n=10$)	P
Age (years)	38 ± 8	37 ± 4	38 ± 10	0.85
Male	17 (85)	9 (90)	8 (80)	0.71
BMI (kg/m^2)	24.6 ± 4.1	25.3 ± 5.2	23.9 ± 2.8	0.82
Waist circumference (cm)	87.3 ± 8.5	88.1 ± 9.4	86.5 ± 8.0	0.91
Systolic blood pressure (mmHg)	120 ± 11	122 ± 10	117 ± 11	0.16
Diastolic blood pressure (mmHg)	68 ± 7	69 ± 9	67 ± 5	0.45
Heart rate (beats/min)	64 ± 7	65 ± 9	63 ± 6	0.62
Current smoker	5 (25)	2 (20)	3 (30)	0.71
Previous smoker	6 (30)	2 (20)	4 (40)	0.45
Dyslipidemia	9 (45)	5 (50)	4 (40)	0.71
Glucose intolerance	3 (15)	2 (20)	1 (10)	0.71
Metabolic syndrome	1 (5)	0 (0)	1 (10)	0.71

Data are expressed as mean ± SD or n (%). BMI indicates body mass index.

dial %FMD and lipid profiles. Values of $P<0.05$ were considered significant.

3. Results

3.1. Postprandial lipid profiles and endothelial function

Postprandial changes in vital signs, lipid profile and endothelial function before treatment in all 20 subjects are shown in supplementary table (see supplementary data 1). Postprandial serum levels of TG, RLP-C and apoB-48 increased significantly and reached peak levels at the 4th hour (TG: from 115 ± 42 to 240 ± 115 mg/dl; RLP-C: from 5.1 ± 1.8 to 11.7 ± 6.1 mg/dl; apoB-48: from 4.5 ± 2.4 to 9.4 ± 3.8 μg/ml; fasting vs. at 4 h, all $P<0.0001$) and returned to baseline at the 8th hour. Postprandial plasma glucose levels also increased significantly at the 2nd hour (from 92 ± 11 to 113 ± 38 mg/dl, fasting vs. at 2 h, $P<0.005$) and returned to baseline at the 6th hour. In contrast, serum levels of Total-C, LDL-C and MDA-LDL significantly decreased in the postprandial state. Regarding endothelial function, postprandial %FMD decreased significantly and reached the lowest level at the 4th hour (from 8.5 ± 2.1 to 6.0 ± 2.1%, fasting vs. at 4 h, $P<0.0001$) and still remained slightly decreased value at the 8th hour (fasting vs. at 8 h, $P<0.05$). Linear regression analysis revealed that the maximum reduction in postprandial %FMD was significantly associated with the maximum increases in postprandial TG and apoB-48 concentrations (TG: $r=-0.499$, $P<0.05$; RLP-C: $r=-0.410$, $P=0.07$; apoB-48: $r=-0.551$, $P<0.05$; glucose: $r=-0.053$, $P=0.82$).

3.2. Effects of ezetimibe on fasting lipid profiles and endothelial function

Comparison of baseline characteristics in the ezetimibe and control groups is shown in Table 1. No statistically significant differences in clinical characteristics were found between the two groups. During the study, no adverse events occurred and all subjects were available for analysis. Table 2 shows the differences in fasting lipid profile and endothelial function before and after treatment for 4 weeks in the ezetimibe and control groups. Before treatment, laboratory profile and endothelial function assessed by %FMD and %NMD did not differ between the two groups. In the ezetimibe group, fasting levels of Total-C, LDL-C, MDA-LDL, TG, RLP-C and apoB-48 decreased significantly after treatment for 4 weeks, but lipid profile was not changed after 4 weeks in the control group. Among fasting laboratory data after treatment for 4 weeks, serum LDL-C and apoB-48 concentrations were significantly lower in the ezetimibe group than those in the control group. With regard to endothelial function during a fasting state, there was no significant difference between %FMD before treatment and that after treatment for 4 weeks in the ezetimibe group ($P=0.11$) and there was no significant difference

Table 2
Laboratory data and endothelial function during a fasting state before and after treatment for 4 weeks in the ezetimibe and control groups.

Variables	Ezetimibe ($n=10$)			Control ($n=10$)		
	Before	After 4 weeks	P	Before	After 4 weeks	P
Laboratory data						
Total-C (mg/dl)	225 ± 25	184 ± 28	<0.01	208 ± 10	199 ± 13	0.09
LDL-C (mg/dl)	135 ± 20	97 ± 20*	<0.01	128 ± 14	118 ± 20	0.09
HDL-C (mg/dl)	61 ± 12	61 ± 10	0.92	56 ± 17	58 ± 19	0.48
MDA-LDL cholesterol (U/l)	134 ± 23	92 ± 21	<0.01	122 ± 21	115 ± 29	0.34
TG (mg/dl)	116 ± 43	89 ± 25	<0.05	113 ± 44	109 ± 58	0.89
RLP-C (mg/dl)	5.2 ± 1.8	3.8 ± 1.3	<0.05	5.1 ± 1.9	4.5 ± 1.6	0.17
apoB-48 (μg/ml)	4.5 ± 2.9	2.4 ± 1.3*	<0.01	4.4 ± 1.9	3.8 ± 1.0	0.33
Adiponectin (μg/ml)	6.9 ± 2.0	6.8 ± 1.6	0.31	6.2 ± 3.9	6.2 ± 3.2	0.73
Blood glucose (mg/dl)	90 ± 11	93 ± 9	0.08	94 ± 11	94 ± 10	0.80
Insulin (μIU/ml)	8.0 ± 5.5	6.7 ± 3.9	0.14	6.1 ± 2.2	6.5 ± 2.1	0.26
HOMA-IR	1.8 ± 1.2	1.6 ± 0.9	0.24	1.5 ± 0.7	1.5 ± 0.7	0.44
HbA1c (%)	5.0 ± 0.1	5.0 ± 0.1	0.81	5.0 ± 0.5	4.9 ± 0.5	0.08
MCP-1 (pg/ml)	402 ± 521	381 ± 511	0.07	213 ± 51	198 ± 38	0.29
Endothelial function						
Brachial artery diameter (mm)	4.01 ± 0.70	4.04 ± 0.58	0.48	3.83 ± 0.66	3.93 ± 0.64	0.17
%FMD	8.4 ± 1.9	9.2 ± 2.3	0.11	8.5 ± 2.5	7.9 ± 1.9	0.19
%NMD	19.8 ± 5.8	19.4 ± 4.3	0.67	21.9 ± 6.8	20.9 ± 3.0	0.68

Data are expressed as mean ± SD. Total-C, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; MDA, malondialdehyde; TG, triglyceride; RLP-C, remnant lipoprotein cholesterol; apoB-48, apolipoprotein B-48; HOMA-IR, homeostasis model assessment of insulin resistance; MCP, monocyte chemotactic protein; FMD, flow-mediated dilation; NMD, nitroglycerin-mediated dilation.

* $P<0.05$, vs. control group after treatment for 4 weeks.

in %FMD between the two groups after treatment for 4 weeks ($P=0.31$).

3.3. Effects of ezetimibe on postprandial lipid profiles and endothelial function

Comparison of postprandial lipid profiles and endothelial function before and after treatment for 4 weeks in the ezetimibe and control groups is shown in supplementary table (see supplementary data 2). Before treatment, postprandial lipid profiles and endothelial function did not differ between the two groups. After treatment for 4 weeks, the AUC of Total-C, LDL-C and MDA-LDL between 0 and 8 h decreased significantly in the ezetimibe group than those in the control group. Fig. 1 shows the changes in postprandial TRLs and %FMD before and after treatment for 4 weeks in the two groups. Postprandial serum TG, RLP-C and apoB-48 concentrations also decreased significantly in the ezetimibe group (AUC of TG: from 1419 ± 594 to 968 ± 321 mg h/dl, $P<0.05$; RLP-C: from 66.9 ± 27.6 to 38.9 ± 15.4 mg h/dl, $P<0.01$; apoB-48: from 58.8 ± 27.5 to 36.2 ± 17.0 μg h/ml, $P<0.05$), whereas no significant changes were observed in the control group (Fig. 1A–C). Regarding postprandial endothelial function, decrease in postprandial %FMD in the ezetimibe group was suppressed significantly after 4 weeks (from $9.2 \pm 2.3\%$ to $8.1 \pm 2.3\%$ at the 4th hour), whereas decrease in postprandial %FMD in the control group after 4 weeks was almost in the same range as that before treatment (from $7.9 \pm 1.9\%$ to $5.7 \pm 2.0\%$ at the 4th hour) (Fig. 1D). The maximum change in postprandial %FMD decreased significantly after 4 weeks in the ezetimibe group (from $-2.6 \pm 1.1\%$ to $-1.2 \pm 0.8\%$, $P<0.05$) but not different in the control group (from $-2.5 \pm 0.4\%$ to $-2.3 \pm 1.0\%$, $P=0.14$) (Fig. 1D).

4. Discussion

In the present study, postprandial hyperlipemia induced by a conventional oral cookie loading test, a real-life situation of ingestion of dietary fat, was shown to be closely related to transient postprandial endothelial dysfunction. To the best of our knowledge, the present study is the first study showing that ezetimibe monotherapy improves postprandial hyperlipemia-induced endothelial dysfunction.

Methods for measuring apoB-48, a specific marker of intestinal lipoproteins, have recently been developed, and these methods have enabled analysis of the particle numbers of exogenous lipoproteins. Several studies have demonstrated that fasting and postprandial apoB-48 levels were significantly higher in obese and hyperlipidemic subjects or in subjects with metabolic syndrome [17]. Moreover, impaired postprandial metabolism of apoB-48 has been shown to lead to atherosclerosis in rats with metabolic syndrome [18]. Cohn et al. demonstrated that postprandial increase in TRL-TG level was mainly due to an increase in apoB48-containing TRL in normolipidemic male subjects, indicating that intestinal lipoproteins containing apoB-48 may predominantly contribute to postprandial atherogenic conditions [19]. In fact, strong relationships were observed between postprandial TG, apoB-48 increase and FMD impairment in the present study. These findings are of considerable interest, since one could argue that exogenous TG-rich lipoproteins may play a dominant role in the pathogenesis of postprandial endothelial dysfunction.

An oral cookie loading test induces not only hyperlipemia but also hyperglycemia. Postprandial hyperglycemia also induces endothelial dysfunction, especially in patients with diabetes or glucose intolerance, and increase in glucose level (glucose spike) is closely associated with impairment of postprandial endothelial function [20,21]. It has been shown that secretion of insulin induced by postprandial hyperglycemia stimulates synthesis of endothelin-1, a central vasoconstrictive hormone, and reduces nitric oxide production [22]. The present study revealed close correlation between postprandial FMD impairment and increase in postprandial TRLs, but not glucose. This observation might be due to the enrolled subjects, among which only 3 subjects had glucose intolerance, and indicates the possibility that postprandial hyperlipemia occurs more commonly than postprandial hyperglycemia in general populations in daily life.

As was found in a previous study [10], our study showed that ezetimibe decreases fasting and postprandial TG, RLP-C and apoB-48 levels after the fat loading test. Tremblay et al. investigated the effect of ezetimibe on apolipoprotein-B metabolism and showed that ezetimibe decreases the levels of TRL apoB-48 by reduction in the intestinal secretion of TRL apoB-48 in male subjects with mixed hyperlipemia [23]. Additionally, the molecular mechanisms of ezetimibe-induced attenuation of postprandial hyperlipemia have recently been elucidated by using mouse models of metabolic syndrome in which ezetimibe inhibits not only cholesterol absorption but also uptake, intracellular trafficking and metabolism of long-chain fatty acids in enterocytes, resulting in reduction of the formation of TG and apoB-48-containing lipoproteins in the small intestine [24]. These findings support our concept that ezetimibe markedly decreases the levels of postprandial TRLs, resulting in prevention of postprandial lipemia-induced endothelial dysfunction.

Only one previous study by Olijhoek et al. showed that combination therapy with low-dose simvastatin and ezetimibe preserved post-fat load endothelial function contrary to high-dose simvastatin monotherapy in male metabolic syndrome patients [16]. The present study is the first study to demonstrate that ezetimibe monotherapy preserved post-fat load endothelial function in a general population. Several studies have been performed to examine the effects of lipid-lowering drugs including statins and fibrates on postprandial hyperlipemia [25] and lipemia-induced endothelial dysfunction [26,27]. Most of those studies showed that both statins and fibrates have beneficial effects on postprandial hyperlipemia and lipemia-induced endothelial dysfunction, possibly due to direct anti-inflammatory and anti-oxidant effects as well as the lipid-lowering actions of the drugs. In some previous studies, it has been shown that ezetimibe monotherapy or combination therapy with a statin also reduces inflammatory and oxidative stress markers [28,29]. In addition, in an animal study using apoE knockout mice, ezetimibe has been shown to increase the production of endothelial nitric oxide synthase (eNOS) and decrease the production of interleukin-6, resulting in improvement of endothelial function [30]. In the present study, we did not examine the effect of ezetimibe on postprandial inflammatory or oxidative status. Moreover, we did not make a direct comparison between ezetimibe and other lipid-lowering agents. Therefore, we could not conclude whether the beneficial effects of ezetimibe on postprandial hyperlipemia and lipemia-induced endothelial dysfunction surpass the effects of other lipid-lowering agents and whether the administration of ezetimibe improves postprandial inflammation and oxidative stress. However, its lipid-lowering action may be one of the potential mechanisms by which ezetimibe improves postprandial endothelial dysfunction. Our findings support the concept that beneficial effects of ezetimibe on postprandial hyperlipemia and lipemia-induced endothelial dysfunction will prevent or delay the development of atherosclerosis. Further large-scale, prospective trials are needed to assess the relationship between the lipid-lowering effect of ezetimibe on postprandial hyperlipemia and the incidence of future cardiovascular events.

4.1. Study limitations

There are several important limitations of our study. First, this study was open-label study and the number of subjects enrolled

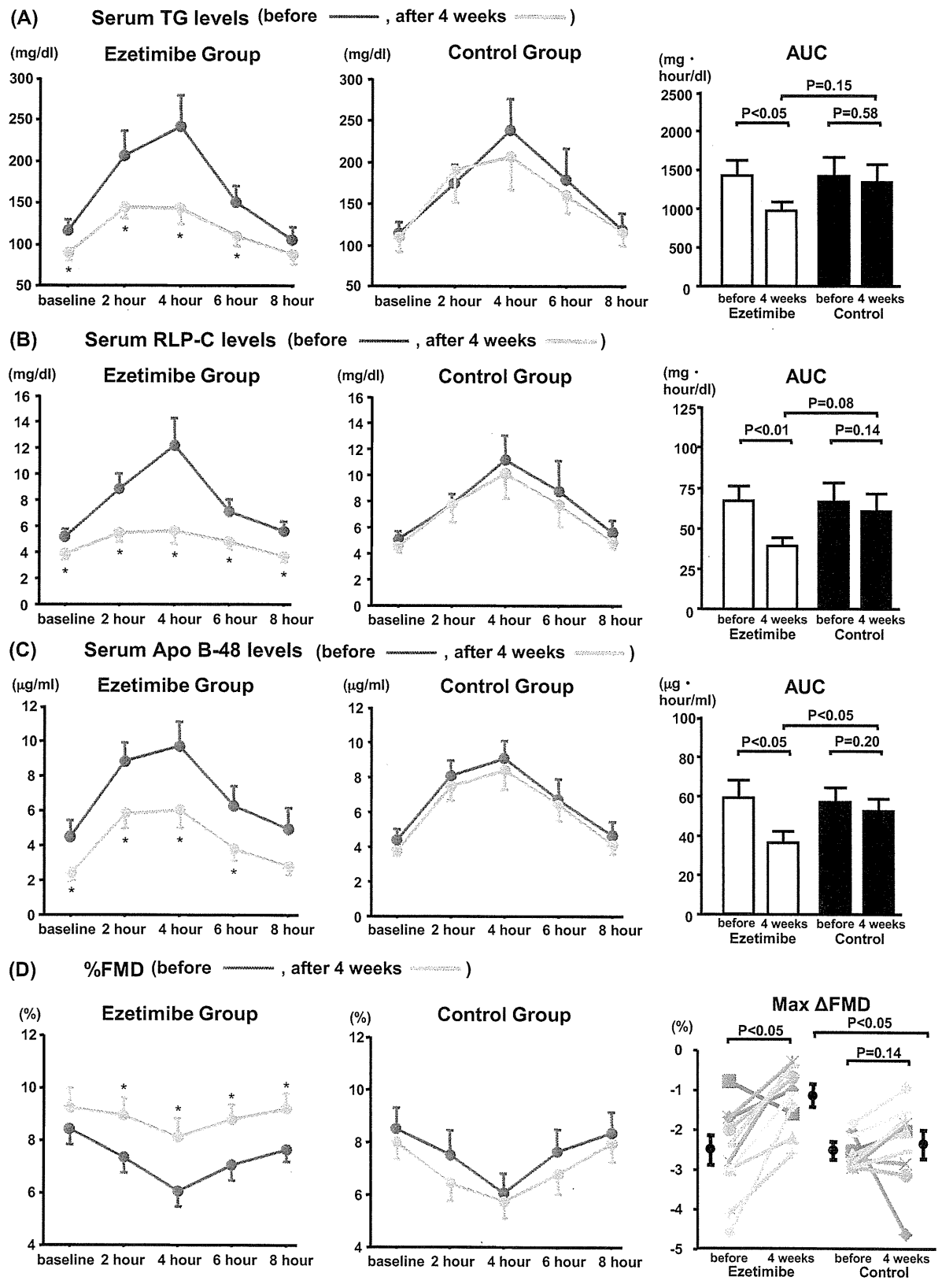

Fig. 1. (A–C) Postprandial changes in serum TG, RLP-C and apoB-48 levels and the AUC for postprandial serum TG, RLP-C and apoB-48 levels before and after treatment for 4 weeks in the ezetimibe and control groups (*open bar* indicates ezetimibe group; *solid bar*, control group). (D) Postprandial changes in %FMD and changes in maximum reduction of %FMD after the cookie test (max Δ%FMD) before and after treatment for 4 weeks in the ezetimibe and control groups. Data are expressed as mean ± SE. $^{*}P < 0.05$, vs. before treatment.

in our study was small, therefore a degree of selection bias might have occurred. Second, we did not examine postprandial insulin concentrations or postprandial inflammatory and oxidative stress status, which are well known to affect eNOS transcription and/or the release of vasoconstrictive mediators, resulting in endothelial dysfunction. Meal absorption is a complex phenomenon that involves the interaction of many factors; therefore, these parameters may need to be measured to improve test reliability and to establish the efficacy of ezetimibe for postprandial status. Third, a method for assessment of postprandial hyperlipemia has not been established, and various fat loading tests, such as oral fat meal, fat cream intake and intravenous fat load, have been performed in previous studies. Cookies are considered as a natural daily food or meal. Moreover, it has been shown by Harano et al. that the cookie test provided sufficient information about glucose intolerance and postprandial hyperlipemia [13]. Thus the oral cookie loading test with a definite quantity of fat per body surface area may be a reliable method for detecting postprandial metabolic disturbances.

5. Conclusions

The present study demonstrated that postprandial hyperlipemia is significantly associated with transient endothelial dysfunction and clearly showed that ezetimibe improves postprandial hyperlipemia and lipemia-induced endothelial dysfunction. Measurements of postprandial parameters may be useful and reliable for selecting patients with postprandial metabolic disturbances, and ezetimibe may be a potent agent for improving postprandial hyperlipemia.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.04.019.

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