ORIGINAL RESEARCH

Determinants of Myocardial Lactate Production During Acetylcholine Provocation Test in Patients With Coronary Spasm

Koichi Kaikita, MD, PhD; Masanobu Ishii, MD; Koji Sato, MD, PhD; Masafumi Nakayama, MD, PhD; Yuichiro Arima, MD, PhD; Tomoko Tanaka, MD, PhD; Koichi Sugamura, MD, PhD; Kenji Sakamoto, MD, PhD; Yasuhiro Izumiya, MD, PhD; Eiichiro Yamamoto, MD, PhD; Kenichi Tsujita, MD, PhD; Megumi Yamamuro, MD, PhD; Sunao Kojima, MD, PhD; Hirofumi Soejima, MD, PhD; Seiji Hokimoto, MD, PhD; Kunihiko Matsui, MD, MPH; Hisao Ogawa, MD, PhD

***Background*—**Myocardial lactate production in the coronary circulation during acetylcholine (ACh)-provocation test (abbreviated as lactate production) provides supporting evidence for coronary spasm–induced myocardial ischemia. The purpose of this study was to examine the clinical features, predictive factors, and prognosis of patients with coronary vasospastic angina (VSA) and lactate production.

***Methods and Results*—**We examined all 712 patients who underwent both myocardial lactate measurement during ACh-provocation test in the left coronary artery and genetic screening test of a –786T/C polymorphism in the 5′-flanking region of the endothelial nitric oxide synthase (*eNOS*) gene between January 1991 and December 2010. Lactate production was observed in 252 of the 712 patients and in 219 of 356 VSA patients diagnosed by ACh-provocation test. Compared with lactate production–negative VSA patients, the lactate production–positive counterparts were more likely to be nonsmoker female diabetics with –786T/C *eNOS* polymorphism (61% vs 31%, $P<0.001$, 62% vs 34%, $P<0.001$, 24% vs 14%, $P=0.016$, and 25% vs 15%, $P=0.018$, respectively). Multivariable logistic regression analysis identified female sex, diabetes mellitus, and –786T/C *eNOS* polymorphism to correlate with lactate production (odds ratio 3.51, 95% CI 2.16 to 5.70, $P<0.001$; odds ratio 2.53, 95% CI 1.38 to 4.65, $P=0.003$; and odds ratio 1.85, 95% CI 1.02 to 3.35, $P=0.044$, respectively). Kaplan–Meier survival curve showed no difference in 5-year survival rate free from major adverse cardiac events between lactate production–positive and –negative VSA patients ($P=0.319$).

***Conclusions*—**The results indicated that female sex, diabetes, and mutation in –786T/C *eNOS* gene correlate with ACh-provoked myocardial ischemia in patients with coronary spasm. **(*J Am Heart Assoc*. 2015;4:e002387 doi: 10.1161/JAHA.115.002387)**

Key Words: acetylcholine-provocation test • coronary spasm • endothelial nitric oxide synthase polymorphism • myocardial lactate production

Coronary spasm plays an important role in the pathogenesis of acute and chronic coronary heart diseases.[1–10] Because coronary vasospastic angina (VSA) can be diagnosed in only ≈20% of the patients who present with spontaneous attack and ischemic electrocardiogram (ECG) changes,[11] the spasm-provocation test with acetylcholine (ACh) or ergonovine is widely used for the diagnosis of VSA.[12–20] While epicardial coronary spasm can be viewed angiographically during the ACh-provocation test, the test does not confirm the presence of myocardial ischemia. For this reason, measurement of myocardial lactate production in the coronary circulation is used as supporting diagnostic marker in the evaluation of coronary spasm–induced myocardial ischemia during ACh-provocation test in the left coronary artery (*for simplicity, this is abbreviated in the text to* "lactate production").

ACh-induced vasodilatation is thought to be mediated by nitric oxide (NO) released from the endothelium.[21,22] In patients with coronary spasm, the basal tone of the coronary arteries is increased, and the hyperreactivity to nitrovasodilators seen in patients with coronary spasm is consistent with decreased endothelial release of NO.[21,23] We have reported previously the presence of –786T/C polymorphism in the 5′-flanking region of the endothelial NO synthase (*eNOS*) gene and highlighted its association with coronary spasm.[24] We argued that *eNOS* gene mutation could reduce endothelial NO

From the Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan (K.K., M.I., K. Sato, M.N., Y.A., T.T., K. Sugamura, K. Sakamoto, Y.I., E.Y., K.T., M.Y., S.K., H.S., S.H., H.O.); Department of Community Medicine, Kumamoto University Hospital, Kumamoto, Japan (K.M.).

Correspondence to: Koichi Kaikita, MD, PhD, Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kumamoto University, 1-1-1, Honjo, Chuo-ku, Kumamoto 860-8556, Japan. E-mail: kaikitak@kumamoto-u.ac.jp

Received July 9, 2015; accepted October 23, 2015.

synthesis and predispose patients with the mutation to coronary spasm.[24] In another study, we also demonstrated the presence of a low lactate extraction ratio during the ACh-provocation test in patients with coronary spasm harboring –786T/C polymorphism in the *eNOS* gene.[25] To our knowledge, however, there is no information on the factors that contribute to lactate production (eg, *eNOS* gene polymorphism) in the general population. The purpose of the present study was to investigate the clinical features, predictive factors, and long-term prognosis of VSA patients with the lactate production.

Methods

Study Population and ACh-Provocation Test

We analyzed retrospectively the angiographic coronary vasomotor response induced by ACh injection in 1877 consecutive patients who had typical and atypical angina-like chest pain and were admitted to Kumamoto University Hospital between January 1991 and December 2010. Among these, we excluded 117 patients for the following reasons: acute myocardial infarction (n=20), cardiomyopathy (n=75), Brugada syndrome (n=10), and other conditions (n=12). After the exclusion, data for 1760 patients who had undergone selective ACh-provocation test were analyzed. They included all 712 patients who also consented to genetic screening for *eNOS* gene –786T/C polymorphism.

The risk factors for coronary artery disease were defined as current smoking (smoking within 1 year), hypertension (>140/90 mm Hg or taking antihypertensive medications), dyslipidemia (high-density lipoprotein cholesterol <40 mg/dL, low-density lipoprotein cholesterol ≥140 mg/dL, or triglycerides ≥150 mg/dL or taking medications for dyslipidemia), diabetes mellitus (symptoms of diabetes plus casual plasma glucose concentration ≥200 mg/dL, fasting plasma glucose concentration ≥126 mg/dL, 2-hour plasma glucose concentration ≥200 mg/dL during 75 g oral glucose tolerance test, or taking medications for diabetes mellitus), and family history of ischemic heart disease (IHD), including obstructive coronary artery disease, VSA, and myocardial infarction.

The ACh-provocation test was performed as described previously in "Guidelines for Diagnosis and Treatment of Patients With Vasospastic Angina" by the Japanese Circulation Society.[26] Coronary spasm was defined as total or subtotal obstruction within the borders of one isolated coronary segment as defined by the American Heart Association,[27] or

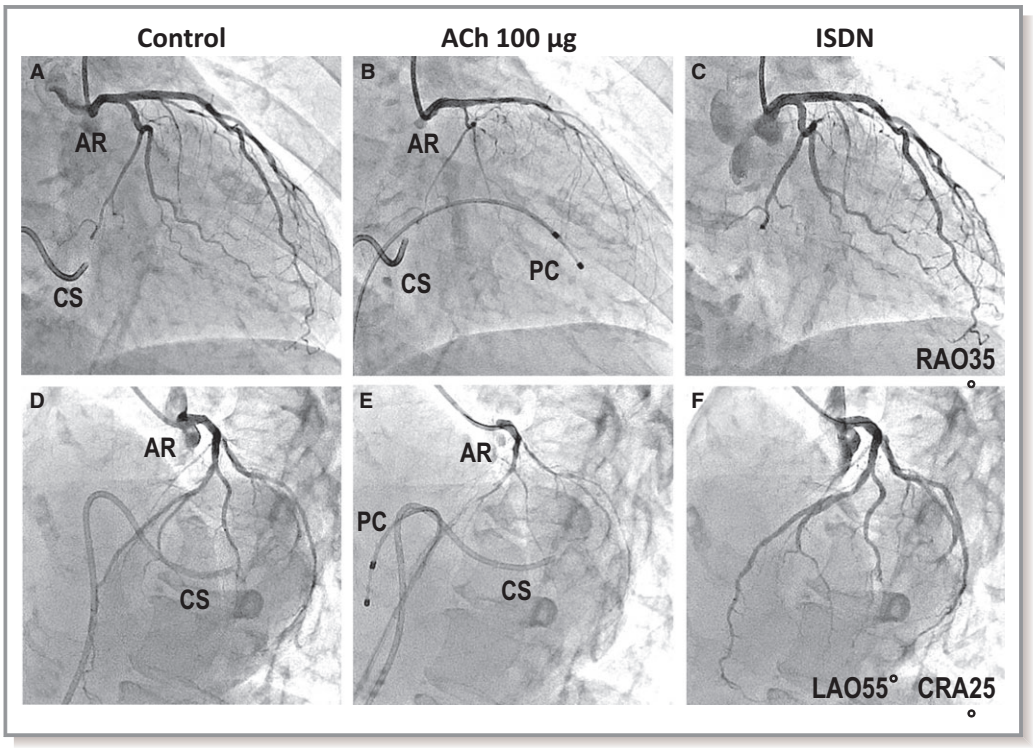

Figure 1. ACh-induced coronary spasm and sampling source for serum lactate at the AR and CS in the coronary circulation. A through C, CAG at RAO35°. D through F, CAG at LAO55° RAO35°. B and E, Injection of 100 μg ACh into the left coronary artery induced diffuse spasm in the entire left coronary artery. ACh indicates acetylcholine; AR, aortic root; CS, coronary sinus; PC, pacing catheter; ISDN, isosorbide dinitrate.

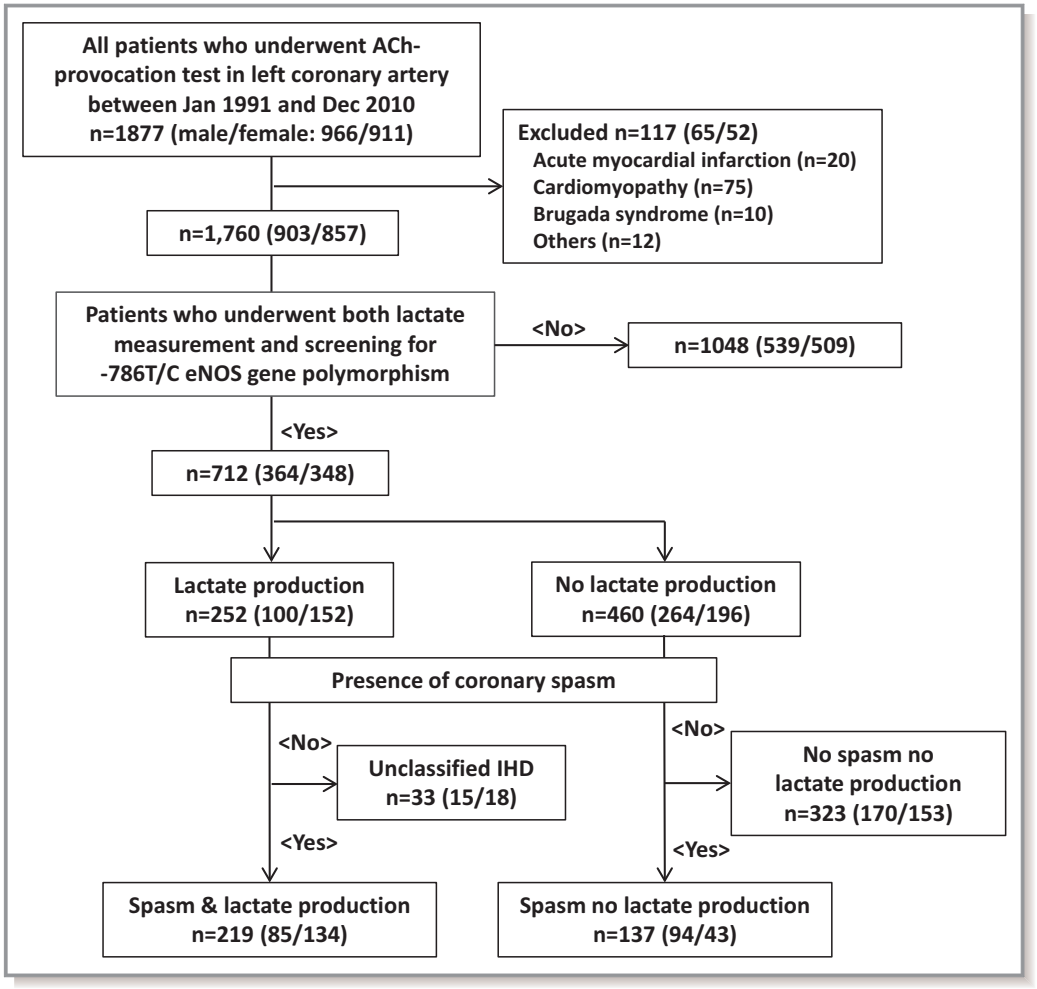

Figure 2. Flow chart of the study recruitment process. ACh indicates acetylcholine; eNOS, endothelial nitric oxide synthase; IHD, ischemic heart disease.

severe diffuse vasoconstriction observed in >2 adjacent coronary segments of epicardial coronary arteries associated with transient myocardial ischemia, as evidenced by ischemic ST-segment changes on the ECG. In this study, ischemic ST-segment changes were defined as ST-segment elevation (>0.1 mV), ST-segment depression (>0.1 mV) from baseline level occurring at 60 to 80 ms after the J point in ≥2 contiguous leads on the 12-lead ECG, or appearance of a new negative U-wave on ECG.

Lactate production was estimated by measuring serum lactate concentrations at the root of the aorta (AR) and coronary sinus (CS), sampled during myocardial ischemia induced by ACh-provocation (see Figure 1). The lactate production ratio was calculated by using the following formula: $\{[(\text{lactate}_{AR}-\text{lactate}_{CS})/\text{lactate}_{AR}]\times 100\%\}$. The ratio is positive in healthy subjects,[28] while a negative ratio is a definite marker of myocardial ischemia. We usually examine coronary sinus lactate production during the ACh-provocation test only in the left coronary artery, because the great coronary sinus drains blood from the left coronary system but not from the right.

Screening for –786T/C Polymorphism in *eNOS* Gene

An allele-specific oligonucleotide method was used in screening for –786T/C polymorphism in the *eNOS* gene. Hybridization was accomplished with ^{32}P-radiolabeled oligonucleotides corresponding to the probe for either the –786T allele or the –786C allele. Details of the method have been published previously.[24] Briefly, the polymerase chain reaction fragments, 236 bp in length, including the –786T/C polymorphism site, were blotted in duplicate onto nylon membranes. Hybridization was accomplished with ^{32}P-radiolabeled oligonucleotides corresponding to either the –786T sequence (50-GGG TCA GCC AGC CAG GGAA-30; probe for the –786T sequence) or the –786C sequence (50-GGG TCA GCCGGC CAG GGAA-30; probe for the –786C sequence).

The study protocol was approved by the Human Ethics Review Committee of Kumamoto University, and a signed consent form was obtained from each subject.

Follow-up Data

Follow-up data were obtained directly from the patients, their families, or their family physicians, in addition to the information available on the medical records. Major adverse cardiac events (MACEs) were assessed by physicians blinded to the medical details available in the medical records. The primary end point was MACEs, defined as cardiac death, hospitalization for acute myocardial infarction, and unstable angina pectoris. The time frame in the survival analysis was defined as time from the date of diagnosis to the date of the first event or until December 2012. The secondary end point was all-cause mortality. Cardiac death was defined as sudden death or death associated with acute myocardial infarction. Acute myocardial infarction was defined by the presence of prolonged (>30 minutes) chest pain, associated with ST-segment changes and elevated cardiac enzyme levels. Unstable angina pectoris represented recurrence or worsening of chest discomfort or pain, associated with ischemic ECG changes.

Statistical Analysis

Data for normally distributed continuous variables were expressed as mean±SD, whereas those with skewed distribution were expressed as median values (IQR). Continuous variables were analyzed by using the unpaired *t* test or Mann–Whitney *U* test, as appropriate. Categorical variables were presented by percentage values, and intergroup comparisons were analyzed by using the χ^2 test or Fisher's exact test as appropriate. Age, sex, and the relationships between the results of ACh-provocation test and other significant parameters in simple logistic analysis were entered into multivariable logistic regression analysis by using the forced entry method, and the Hosmer–Lemeshow goodness-of-fit statistic was calculated to assess model calibration. The Breslow–Day test was carried out to confirm the relationship between lactate production and coronary spasm, after controlling for sex. Survival was analyzed by the Kaplan–Meier survival curve with the log-rank test. The multivariable Cox hazard regression analysis was carried out for identification of predictors of outcome. Significant variables according to univariate analysis and found to be involved in VSA outcome were subjected to the forced entry method. A *P* value of <0.05 denoted statistical

Table 1. Clinical Characteristics of 712 Patients Who Underwent Both Lactate Measurement and Screening for –786T/C *eNOS* Gene Polymorphism and the Excluded 1048 Patients

	Patients Included in the Present Study (n=712)	Patients Excluded in the Present Study (n=1048)	*P* Value
Age (SD), y	63.0 (10.6)	63.1 (11.2)	0.957
Female sex, n (%)	348 (49)	509 (49)	0.899
Body mass index (SD), kg/m^2	23.7 (3.2)	23.7 (3.7)	0.795
Current smoking, n (%)	348 (49)	487 (47)	0.330
Diabetes mellitus, n (%)	147 (21)	200 (19)	0.448
Hypertension, n (%)	264 (37)	455 (44)	0.007
Dyslipidemia, n (%)	272 (38)	471 (45)	0.004
Fasting blood glucose [IQR], mg/dL	91 [86–101]	91 [85–101]	0.515
Hemoglobin A1c [IQR], %	5.9 [5.6–6.4]	5.9 [5.6–6.3]	0.323
LDL cholesterol [IQR], mg/dL	110 [90–133]	114 [94–137]	0.012
HDL cholesterol [IQR], mg/dL	50 [42–62]	50 [40–61]	0.263
Triglyceride [IQR], mg/dL	112 [81–155]	114 [82–155]	0.660
hs-CRP [IQR], mg/dL	0.06 [0.05–0.17]	0.08 [0.05–0.24]	0.649
eGFR (SD), mL/min per 1.73 m^2	75.3 (17.7)	72.5 (19.5)	0.005
Epicardial stenosis ≧75%, n (%)	134 (19)	223 (21)	0.208
Coronary spasm, n (%)	356 (50)	499 (48)	0.326

Data are mean (SD), median [IQR], or n (%). eNOS indicates endothelial nitric oxide synthase; LDL, low-density lipoprotein; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate.

Table 2. Clinical Characteristics of ACh-Positive and -Negative Patients

	Entire Group, n=712	ACh Positive, n=356 (50%)	ACh Negative, n=356 (50%)	*P* Value
Age (SD), y	63.0 (10.6)	63.9 (10.2)	62.2 (10.9)	0.029
Female sex, n (%)	348 (49)	177 (50)	171 (48)	0.653
Body mass index (SD), kg/m^2	23.7 (3.2)	23.8 (3.2)	23.6 (3.2)	0.391
Current smoking, n (%)	348 (49)	173 (49)	175 (50)	0.823
Diabetes mellitus, n (%)	147 (21)	72 (20)	75 (21)	0.811
Hypertension, n (%)	264 (37)	142 (40)	122 (34)	0.128
Dyslipidemia, n (%)	272 (38)	155 (44)	117 (33)	0.003
Family history of IHD, n (%)	111 (16)	67 (19)	44 (13)	0.019
Fasting blood glucose [IQR], mg/dL	91 [86–101]	92 [86–103]	91 [85–100]	0.199
Hemoglobin A1c [IQR], %	5.9 [5.6–6.4]	5.8 [5.5–6.2]	6.0 [5.7–6.7]	0.022
LDL cholesterol [IQR], mg/dL	110 [90–133]	110 [90–131]	111 [90–134]	0.554
HDL cholesterol [IQR], mg/dL	50 [42–62]	50 [42–63]	49 [41–61]	0.381
Triglyceride [IQR], mg/dL	112 [81–155]	109 [80–152]	114 [83–157]	0.276
hs-CRP [IQR], mg/dL	0.06 [0.05–0.17]	0.06 [0.05–0.17]	0.07 [0.05–0.17]	0.171
eGFR (SD), mL/min per 1.73 m^2	75.3 (17.7)	74.8 (17.8)	75.7 (17.5)	0.554
Epicardial stenosis ≥75%, n (%)	134 (19)	86 (24)	48 (14)	<0.001
Left ventricular ejection fraction (SD), %	72.8 (9.5)	72.0 (10.4)	73.5 (8.6)	0.048
Lactate production, n (%)	252 (35)	219 (62)	33 (9)	<0.001
eNOS −786T/C mutation, n (%)	126 (18)	75 (21)	51 (14)	0.018
Medications after ACh-provocation test				
CCB, n (%)	498 (70)	332 (93)	166 (47)	<0.001
ACE inhibitor, n (%)	88 (12)	44 (12)	44 (12)	0.989
ARB, n (%)	40 (6)	32 (9)	8 (2)	<0.001
Nitrates, n (%)	87 (12)	64 (18)	23 (7)	<0.001
Nicorandil, n (%)	19 (3)	17 (5)	2 (1)	<0.001
β-Blockers, n (%)	53 (8)	33 (9)	20 (6)	0.062
Statins, n (%)	143 (20)	99 (28)	44 (12.4)	<0.001
Aspirin, n (%)	181 (26)	120 (34)	61 (17)	<0.001

Data are mean (SD), median [IQR], or n (%). Ach indicates acetylcholine; IHD, ischemic heart disease; LDL, low-density lipoprotein; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate; LCA, left coronary artery; CCB, calcium channel blocker; ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; eNOS, endothelial nitric oxide synthase.

significance; all tests were 2-tailed. Statistical analyses were performed by using Statistical Package of the Social Science version 22.0 (SPSS).

Results

Prevalence of ACh-Provoked Coronary Spasm

Figure 2 provides a flow chart of the patient recruitment process. Among the 1877 patients examined between January 1991 and December 2010, data for all 712 consecutive patients (age 63.0±11.0 years) who underwent both myocardial lactate and *eNOS* genetic testing were analyzed. Lactate production and coronary spasm occurred during the provocation test in 252 and 356 patients in the present study, respectively. Patients with (n=252) and without (n=460) lactate production were subdivided according to the presence or absence of coronary spasm. Thus, the 252 patients with lactate production comprised 219 with coronary spasm and 33 with unclassified IHD. The 460 patients without lactate production included 137 with and 323 without coronary spasm (nonspasm group). In other words, ACh-provocation test induced lactate production in 219 of 356 VSA patients (Figure 2).

Clinical Characteristics of the Study Population

Table 1 shows the clinical characteristics of 712 patients who underwent both lactate measurement and screening for –786T/C *eNOS* gene polymorphism and the excluded 1048 patients. The frequencies of hypertension and dyslipidemia and the low-density lipoprotein cholesterol levels were lower and the estimated glomerular filtration rate was higher in the included 712 patients than in the excluded 1048 patients.

Table 2 shows the clinical characteristics of ACh-positive (including spasm patients with [n=219] and without [n=137] lactate production, n=356) and -negative (including patients with unclassified IHD [n=33] and neither spasm [n=323] nor lactate production [n=356]) patients. Age, dyslipidemia, family history of IHD, comorbidity of coronary epicardial stenosis, lactate production, and *eNOS* –786T/C mutation were significantly higher in the ACh-positive than in the ACh-negative group. On the other hand, serum hemoglobin A1c

Table 3. Clinical Characteristics of Lactate Production–Positive and –Negative Patients

	Lactate Production Positive, n=252 (35%)	Lactate Production Negative, n=460 (65%)	*P* Value
Age (SD), y	63.2 (10.3)	62.9 (10.8)	0.769
Female sex, n (%)	152 (60)	196 (43)	<0.001
Body mass index (SD), kg/m^2	23.9 (3.3)	23.5 (3.1)	0.103
Current smokers, n (%)	99 (39)	294 (55)	<0.001
Diabetes mellitus, n (%)	62 (25)	85 (19)	0.047
Hypertension, n (%)	362 (42)	289 (38)	0.079
Dyslipidemia, n (%)	104 (41)	168 (37)	0.198
Family history of IHD, n (%)	42 (17)	69 (15)	0.567
Systolic blood pressure (SD), mm Hg	128.0 (19.1)	127.7 (18.2)	0.860
Diastolic blood pressure (SD), mm Hg	73.1 (12.1)	74.6 (11.0)	0.113
Fasting blood glucose, mg/dL [IQR]	93 [85–106]	92 [85–104]	0.074
Hemoglobin A1c, % [IQR]	5.9 [5.5–6.3]	5.9 [5.6–6.3]	0.962
LDL cholesterol, mg/dL [IQR]	103 [84–124]	112 [90–133]	0.174
HDL cholesterol, mg/dL [IQR]	53 [44–65]	49 [40–62]	0.125
Triglyceride, mg/dL [IQR]	103 [84–124]	123 [77–163]	0.073
hs-CRP, mg/dL [IQR]	0.05 [0.04–0.14]	0.08 [0.05–0.19]	0.694
eGFR (SD), mL/min per 1.73 m^2	75.7 (17.9)	75.0 (17.6)	0.629
Epicardial stenosis ≥75%, n (%)	53 (21)	81 (18)	0.264
Left ventricular ejection fraction (SD), %	72.4 (10.9)	73.0 (8.7)	0.468
LCA spasm-positive, n (%)	219 (87)	137 (30)	<0.001
–786T/C *eNOS* mutation, n (%)	57 (23)	69 (15)	<0.011
Medications after ACh-provocation test			
CCB, n (%)	212 (84)	286 (62)	<0.001
ACE inhibitor, n (%)	35 (14)	53 (12)	0.376
ARB, n (%)	24 (10)	16 (4)	0.001
Nitrates, n (%)	36 (14)	51 (11)	0.225
Nicorandil, n (%)	12 (5)	7 (2)	0.011
β-Blockers, n (%)	26 (10)	27 (6)	0.033
Statins, n (%)	60 (24)	83 (18)	0.073
Aspirin, n (%)	70 (28)	111 (24)	0.308

Data are mean (SD), median [IQR], or n (%). IHD indicates ischemic heart disease; LDL, low-density lipoprotein; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate; LCA, left coronary artery; ACh, acetylcholine; CCB, calcium channel blocker; ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; eNOS, endothelial nitric oxide synthase.

level, triglyceride level, and left ventricular ejection fraction measured with left ventriculography were significantly lower in the ACh-positive than in the ACh-negative group.

Clinical Characteristics and Predictive Factors of Lactate Production

Table 3 shows the clinical characteristics of lactate production–positive (n=252, 35%) and –negative patients (n=460, 65%). Compared with lactate production–negative patients, lactate production–positive patients were more likely to be females, nonsmokers, diabetic, and spasm positive and to have –786T/C *eNOS* polymorphism (60% vs 43%, $P<0.001$; 61% vs 45%, $P<0.001$; 25% vs 19%, $P=0.047$; 87% vs 30%, $P<0.001$; 23% vs 15%, $P=0.011$, respectively). Table 4 shows the results of simple and multivariable regression analyses for lactate production by using data for all 712 patients. Simple logistic regression analysis demonstrated that female sex (odds ratio [OR] 2.05, 95% CI 1.50 to 2.80, $P<0.001$), current smoking (OR 0.54, 95% CI 0.40 to 0.74, $P<0.001$), diabetes mellitus (OR 1.46, 95% CI 1.00 to 2.11, $P=0.048$), ACh-induced coronary spasm (OR 15.6, 95% CI 10.3 to 23.7, $P<0.001$), and *eNOS* –786T/C mutation (OR 1.66, 95% CI 1.12 to 2.45, $P=0.011$) correlated significantly with lactate production. Because the correlation coefficient matrix indicated that sex and smoking habit showed strong correlation ($|r|=0.710$), we excluded smoking habit from the list of variables. Multivariable logistic regression analysis identified female sex (OR 3.01, 95% CI 2.02 to 4.47, $P<0.001$), diabetes mellitus (OR 2.18, 95% CI 1.34 to 3.54, $P=0.002$), and ACh-induced coronary spasm (OR 18.5, 95% CI 11.8 to 28.9, $P<0.001$) as significant predictors of lactate production (Table 4). Hosmer–Lemeshow goodness-of-fit χ^2 value was 8.278 with $P=0.407$. Further, we analyzed the relationship between lactate production and coronary spasm, after controlling for sex. Breslow–Day test for homogeneity of the OR χ^2 values was 4.864 with $P=0.027$, suggesting effect modification by sex. The results of this statistical analysis confirmed the effect of female sex on lactate production.

Table 4. Results of Simple and Multivariable Regression Analyses for ACh-Induced Myocardial Lactate Production in the Study Population

	Simple Regression Analysis			Multivariable Regression Analysis		
	OR	95% CI	*P* Value	OR	95% CI	*P* Value
Age	1.00	0.99–1.02	0.769			
Female sex	2.05	1.50–2.80	<0.001	3.01	2.02–4.47	<0.001
Body mass index (≥25 kg/m^2)	0.97	0.69–1.36	0.864			
Current smoking	0.54	0.40–0.74	<0.001			
Diabetes mellitus	1.46	1.00–2.11	0.048	2.18	1.34–3.54	0.002
Hypertension	1.33	0.97–1.82	0.080	1.23	0.83–1.83	0.299
Dyslipidemia	1.23	0.90–1.69	0.198			
Family history of IHD	1.13	0.74–1.72	0.567			
Systolic blood pressure, mm Hg	1.00	0.99–1.01	0.860			
Diastolic blood pressure, mm Hg	0.99	0.98–1.00	0.113			
Fasting blood glucose, mg/dL	1.00	1.00–1.01	0.559			
Hemoglobin A1c, %	0.87	0.70–1.08	0.211			
LDL-C, mg/dL	1.00	0.99–1.00	0.223			
HDL-C, mg/dL	1.00	1.00–1.01	0.375			
Triglyceride, mg/dL	1.00	1.00–1.00	0.457			
hs-CRP, mg/dL	1.05	0.75–1.49	0.766			
eGFR	0.74	0.47–1.17	0.194			
Left ventricular ejection fraction <50%	3.16	0.65–15.4	0.156			
Epicardial stenosis ≥75%	1.25	0.85–1.83	0.264			
Spasm-positive	15.6	10.3–23.7	<0.001	18.5	11.8–28.9	<0.001
–786T/C *eNOS* mutation	1.66	1.12–2.45	0.011	1.36	0.83–2.22	0.22

ACh indicates acetylcholine; IHD, ischemic heart disease; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol hs-CRP, high-sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate; eNOS, endothelial nitric oxide synthase; OR, odds ratio.

Table 5. Clinical Characteristics of Patients With Coronary Spasm With and Without Myocardial Lactate Production

	Patients With Coronary Spasm		
	Lactate Production Positive, n=219 (62%)	Lactate Production Negative, n=137 (38%)	*P* Value
Age (SD), y	63.6 (10.2)	64.4 (10.3)	0.505
Female sex, n (%)	134 (61)	43 (31)	<0.001
Body mass index (SD), kg/m^2	23.9 (3.4)	23.6 (2.9)	0.528
Current smoking, n (%)	83 (38)	90 (66)	<0.001
Diabetes mellitus, n (%)	53 (24)	19 (14)	0.016
Hypertension, n (%)	90 (41)	52 (38)	0.556
Dyslipidemia, n (%)	94 (43)	61 (45)	0.795
Family history of IHD, n (%)	39 (18)	28 (20)	0.550
Systolic blood pressure (SD), mm Hg	126.9 (18.4)	125.5 (17.4)	0.460
Diastolic blood pressure (SD), mm Hg	72.3 (12.0)	73.8 (11.4)	0.233
Fasting blood glucose, mg/dL [IQR]	94 [86–106]	91 [84–101]	0.074
Hemoglobin A1c, % [IQR]	5.9 [5.5–6.3]	5.8 [5.5–6.0]	0.037
LDL cholesterol, mg/dL [IQR]	104 [84–125]	108 [92–132]	0.568
HDL cholesterol, mg/dL [IQR]	53 [44–65]	52 [40–63]	0.238
Triglyceride, mg/dL [IQR]	101 [77–162]	120 [72–157]	0.336
hs-CRP, mg/dL [IQR]	0.05 [0.04–0.12]	0.05 [0.03–0.17]	0.673
eGFR (SD), mL/min per 1.73 m^2	75.5 (18.2)	73.8 (17.2)	0.407
Epicardial stenosis ≥75%, n (%)	48 (22)	38 (28)	0.212
Left ventricular ejection fraction (SD), %	71.7 (11.4)	72.5 (8.5)	0.486
Diffuse spasm, n (%)	101 (46)	49 (36)	0.054
Multivessel spasm, (%)	57 (26.0)	24 (17.5)	0.062
–786T/C *eNOS* mutation, n (%)	55 (25.1)	20 (14.6)	0.018
Medications after ACh-provocation test			
CCB, n (%)	200 (91)	132 (96)	0.066
ACE inhibitor, n (%)	29 (13)	15 (11)	0.555
ARB, n (%)	22 (10)	10 (7)	0.400
Nitrates, n (%)	34 (16)	30 (22)	0.112
Nicorandil, n (%)	11 (5)	6 (4)	0.805
β-Blockers, n (%)	22 (10)	11 (8)	0.551
Statins, n (%)	57 (26)	42 (31)	0.301
Aspirin, n (%)	67 (31)	53 (39)	0.094

Data are mean (SD), median [IQR], or n (%). IHD indicates ischemic heart disease; LDL, low-density lipoprotein; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate; eNOS, endothelial nitric oxide synthase; ACh, acetylcholine; CCB, calcium channel blocker; ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.

Clinical Characteristics and Predictive Factors of Patients With Coronary Spasm With and Without Lactate Production

Table 5 details the clinical characteristics of the lactate production–positive (n=219, 62%) and –negative patients with coronary spasm (n=137, 38%) (Figure 2). Compared with lactate production–negative spasm patients, the lactate production–positive spasm patients were more likely to be females, nonsmokers, and diabetic and to have *eNOS* –786T/C polymorphism (61% vs 31%, *P*<0.001; 62% vs 34%, *P*<0.001; 24% vs 14%, *P*=0.016; and 25% vs 15%, *P*=0.018, respectively). As shown in Table 6, simple logistic regression analysis demonstrated that female sex (OR 3.45, 95% CI 2.19 to 5.41, *P*<0.001), current smoking (OR 0.32, 95% CI 0.21 to 0.50, *P*<0.001), diabetes mellitus (OR 2.01, 95% CI 1.13 to

Table 6. Results of Simple and Multivariable Regression Analyses for ACh-Induced Myocardial Lactate Production in Patients With Coronary Spasm

	Simple Regression Analysis			Multivariable Regression Analysis		
	OR	95% CI	*P* Value	OR	95% CI	*P* Value
Age	0.99	0.97–1.01	0.503			
Female sex	3.45	2.19–5.41	<0.001	3.51	2.16–5.70	<0.001
Body mass index (≥25 kg/m^2)	0.89	0.56–1.43	0.635			
Current smoking	0.32	0.21–0.50	<0.001			
Diabetes mellitus	2.01	1.13–3.57	0.018	2.53	1.38–4.65	0.003
Hypertension	1.14	0.74–1.77	0.556			
Dyslipidemia	0.94	0.61–1.45	0.795			
Epicardial stenosis ≥75%	0.73	0.45–1.20	0.213			
Diffuse spasm	1.54	0.99–2.38	0.055	1.08	0.66–1.76	0.759
Multivessel spasm	1.66	0.97–2.83	0.064	1.28	0.72–2.28	0.406
−786T/C *eNOS* mutation	1.96	1.12–3.45	0.019	1.85	1.02–3.35	0.044

ACh indicates acetylcholine; eNOS, endothelial nitric oxide synthase; OR, odds ratio.

3.57, *P*=0.018), and *eNOS* −786T/C mutation (OR 1.96, 95% CI 1.12 to 3.45, *P*=0.019) correlated significantly with lactate production in patients with coronary spasm. Because the correlation coefficient matrix indicated that sex and smoking habit showed strong correlation ($|r|$=0.710), we excluded smoking habit from the list of variables. Multivariable logistic regression analysis identified female sex (OR 3.51, 95% CI 2.16 to 5.70, *P*<0.001), diabetes mellitus (OR 2.53, 95% CI 1.38 to 4.65, *P*=0.003), and −786T/C *eNOS* polymorphism (OR 1.85, 95% CI 1.02 to 3.35, *P*=0.044) to correlate with lactate production in patients with coronary spasm. Hosmer–Lemeshow goodness-of-fit χ^2 was 5.076 with *P*=0.651.

Clinical Outcome of VSA Patients Diagnosed by ACh-Provocation Test

During a mean follow-up period of 47±19 months, MACEs were registered in 15 patients (cardiac death, n=0; myocardial infarction, n=3; and unstable angina, n=12). Noncardiac deaths were recorded in 10 patients. The 5-year survival rates free from MACEs and all-cause death were 95.8% and 97.2%, respectively. Kaplan–Meier survival curve showed no difference in 5-year survival rates free from MACEs between lactate production–positive and –negative VSA patients (*P*=0.319 by log-rank test).

Discussion

The present study described the clinical features, predictive factors, and long-term prognosis of VSA patients with lactate production in a large study population. The results showed that myocardial ischemia is more likely to be provoked by ACh in female diabetics with −786T/C *eNOS* polymorphism who present with VSA. The results also showed that despite these differences, the Kaplan–Meier survival analysis showed no difference in 5-year survival rates free from MACEs between lactate production–positive and –negative VSA patients. To the best of our knowledge, this is the first report that describes the relation between ACh-provoked myocardial ischemia and various predictors including sex and genetic factors in a large study population.

Multivariable regression analysis identified female sex, diabetes mellitus, and spasm-positive findings as significant predictors for lactate production by using the data from 712 patients. Actually, 87% of lactate production–positive patients exhibited ACh-provoked coronary spasm in the left coronary artery, whereas 30% of lactate production–negative patients exhibited coronary spasm during the same test. These results suggest that lactate production could be a useful diagnostic tool for myocardial ischemia during the ACh-provocation test. It is not clear at present why angiographically evident coronary spasm did not induce lactate production in the remaining 137 of 356 VSA patients. It is possible that methodological (eg, gap in timing of blood sampling during ACh-provocation test) or physiopathological factors (eg, low-grade ACh-provoked myocardial ischemia) were responsible for the lack of increase in serum lactate levels in the coronary circulation.

The present study identified female sex, diabetes mellitus, and −786T/C *eNOS* polymorphism to correlate with lactate production in VSA patients. In this regard, we identified previously −786T/C polymorphism in the *eNOS* gene and

showed that this polymorphism was strongly associated with coronary spasm.[24] Further, the mutated –786T/C gene exhibited significant reduction in *eNOS* gene promoter activity based on luciferase reporter gene assays.[24] In the present study, the frequency of –786T/C *eNOS* mutation was higher in the VSA group compared with the no-spasm group (25% vs 15%, P=0.005, data not shown). Based on these findings, it is possible that –786T/C *eNOS* mutation could cause severe myocardial ischemia during ACh-provocation test, resulting in high serum lactate levels during coronary spasm. However, the presence of both –786T/C *eNOS* mutation and lactate production did not affect the incidence of MACEs in VSA patients (data not shown). In this retrospective study, VSA patients with *eNOS* mutation and lactate production were treated with antianginal drugs, such as calcium channel blockers and long-acting nitrates. Thus, one cannot rule out that such treatment reduced the cardiovascular events in those patients.

Our data could not explain why lactate production in ACh-provocation test was more common in female than in male VSA patients. Further studies are needed to examine this phenomenon. On the other hand, diabetes mellitus is known to be one of the major coronary risk factors and is closely associated with coronary endothelial dysfunction.[29–34] Previous studies reported the presence of endothelial dysfunction and impaired NO release in both type 2 diabetes[29,30] and insulin-dependent diabetes.[31,32] Patients with insulin resistance alone could also experience coronary endothelial dysfunction.[33,34] It is possible that in diabetic patients, impaired NO release induced by coronary endothelial dysfunction can cause coronary spasm and myocardial ischemia, as assessed by lactate production.

The present study has certain limitations. First, the number of patients was relatively small, mainly because some patients did not consent for the genetic screening test of *eNOS* gene –786T/C polymorphism. Second, we found that 33 (4.6%) unclassified IHD patients (of 712 patients) who underwent ACh-provocation test had lactate production without epicardial coronary spasm. Further analysis to define the diagnosis, clinical features, and treatment of those patients is needed. Third, because the study was retrospective in design, we could not examine the MACE rate in ACh-negative patients. However, follow-up of the ACh-negative group will be clinically important to determine the prognosis and need for any medical treatment. Fourth, the outcome analysis had low statistical power because of the small number of events recorded during the follow-up period. A prospective multicenter study in a large study population with longer follow-up period is needed to investigate the clinical features, predictive factors, and prognoses of VSA patients who undergo the ACh-provocation test and measurement of lactate production.

In conclusion, myocardial lactate production in VSA patients during ACh-provocation test was closely associated with female sex, diabetes mellitus, and –786T/C *eNOS* polymorphism, although such production had no impact on the 5-year survival rate free from MACEs. Further large long-term studies are needed to determine the treatment strategy for VSA patients with lactate production during ACh-provocation test and *eNOS* gene mutation.

Sources of Funding

This study was supported in part by Grant-in-Aid for Scientific Research C24591062 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Disclosures

None.

References

1. Yasue H, Omote S, Takizawa A, Nagao M. Coronary arterial spasm in ischemic heart disease and its pathogenesis. A review. *Circ Res*. 1983;52:I147–I152.
2. Bertrand ME, Lablanche JM, Tilmant PY, Thieuleux FA, Delforge MG, Chahine RA. The provocation of coronary arterial spasm in patients with recent transmural myocardial infarction. *Eur Heart J*. 1983;4:532–535.
3. Maseri A, L'Abbate A, Baroldi G, Chierchia S, Marzilli M, Ballestra AM, Severi S, Parodi O, Biagini A, Distante A, Pesola A. Coronary vasospasm as a possible cause of myocardial infarction. A conclusion derived from the study of "preinfarction" angina. *N Engl J Med*. 1978;299:1271–1277.
4. Ong P, Athanasiadis A, Hill S, Vogelsberg H, Voehringer M, Sechtem U. Coronary artery spasm as a frequent cause of acute coronary syndrome: the CASPAR (Coronary Artery Spasm in Patients With Acute Coronary Syndrome) Study. *J Am Coll Cardiol*. 2008;52:523–527.
5. Nakayama N, Kaikita K, Fukunaga T, Matsuzawa Y, Sato K, Horio E, Yoshimura M, Mizobe M, Takashio S, Tsujita K, Kojima S, Tayama S, Hokimoto S, Sakamoto T, Nakao K, Sugiyama S, Kimura K, Ogawa H. Clinical features and prognosis of patients with coronary spasm-induced non-ST-segment elevation acute coronary syndrome. *J Am Heart Assoc*. 2014;3:e000795 doi: 10.1161/JAHA.114.000795.
6. Pristipino C, Beltrame JF, Finocchiaro ML, Hattori R, Fujita M, Mongiardo R, Cianflone D, Sanna T, Sasayama S, Maseri A. Major racial differences in coronary constrictor response between Japanese and Caucasians with recent myocardial infarction. *Circulation*. 2000;101:1102–1108.
7. Yasue H, Touyama M, Kato H, Tanaka S, Akiyama F. Prinzmetal's variant form of angina as a manifestation of alpha-adrenergic receptor-mediated coronary artery spasm: documentation by coronary arteriography. *Am Heart J*. 1976;91:148–155.
8. Yasue H, Touyama M, Shimamoto M, Kato H, Tanaka S. Role of autonomic nervous system in the pathogenesis of Prinzmetal's variant form of angina. *Circulation*. 1974;50:534–539.
9. Ogawa H, Yasue H, Oshima S, Okumura K, Matsuyama K, Obata K. Circadian variation of plasma fibrinopeptide A level in patients with variant angina. *Circulation*. 1989;80:1617–1626.
10. Soejima H, Irie A, Miyamoto S, Kajiwara I, Kojima S, Hokamaki J, Sakamoto T, Tanaka T, Yoshimura M, Nishimura Y, Ogawa H. Preference toward a T-helper type 1 response in patients with coronary spastic angina. *Circulation*. 2003;107:2196–2200.
11. Sato K, Kaikita K, Nakayama N, Horio E, Yoshimura H, Ono T, Ohba K, Tsujita K, Kojima S, Tayama S, Hokimoto S, Matsui K, Sugiyama S, Yamabe H, Ogawa H. Coronary vasomotor response to intracoronary acetylcholine injection, clinical features, and long-term prognosis in 873 consecutive patients with coronary spasm: analysis of a single-center study over 20 years. *J Am Heart Assoc*. 2013;2:e000227 doi: 10.1161/JAHA.113.000227.
12. Inobe Y, Kugiyama K, Morita E, Kawano H, Okumura K, Tomiguchi S, Tsuji A, Kojima A, Takahashi M, Yasue H. Role of adenosine in pathogenesis of

syndrome X: assessment with coronary hemodynamic measurements and thallium-201 myocardial single-photon emission computed tomography. *J Am Coll Cardiol*. 1996;28:890–896.

13. Okumura K, Yasue H, Matsuyama K, Ogawa H, Kugiyama K, Ishizaka H, Sumida H, Fujii H, Matsunaga T, Tsunoda R. Diffuse disorder of coronary artery vasomotility in patients with coronary spastic angina. Hyperreactivity to the constrictor effects of acetylcholine and the dilator effects of nitroglycerin. *J Am Coll Cardiol*. 1996;27:45–52.
14. Sueda S, Kohno H, Fukuda H, Ochi N, Kawada H, Hayashi Y, Uraoka T. Frequency of provoked coronary spasms in patients undergoing coronary arteriography using a spasm provocation test via intracoronary administration of ergonovine. *Angiology*. 2004;55:403–411.
15. Sueda S, Ochi N, Kawada H, Matsuda S, Hayashi Y, Tsuruoka T, Uraoka T. Frequency of provoked coronary vasospasm in patients undergoing coronary arteriography with spasm provocation test of acetylcholine. *Am J Cardiol*. 1999;83:1186–1190.
16. Yasue H, Horio Y, Nakamura N, Fujii H, Imoto N, Sonoda R, Kugiyama K, Obata K, Morikami Y, Kimura T. Induction of coronary artery spasm by acetylcholine in patients with variant angina: possible role of the parasympathetic nervous system in the pathogenesis of coronary artery spasm. *Circulation*. 1986;74:955–963.
17. Ong P, Athanasiadis A, Borgulya G, Mahrholdt H, Kaski JC, Sechtem U. High prevalence of a pathological response to acetylcholine testing in patients with stable angina pectoris and unobstructed coronary arteries. The ACOVA Study (Abnormal COronary VAsomotion in patients with stable angina and unobstructed coronary arteries). *J Am Coll Cardiol*. 2012;59:655–662.
18. Ong P, Athanasiadis A, Borgulya G, Vokshi I, Bastiaenen R, Kubik S, Hill S, Schäufele T, Mahrholdt H, Kaski JC, Sechtem U. Clinical usefulness, angiographic characteristics, and safety evaluation of intracoronary acetylcholine provocation testing among 921 consecutive white patients with unobstructed coronary arteries. *Circulation*. 2014;129:1723–1730.
19. Takagi Y, Yasuda S, Takahashi J, Tsunoda R, Ogata Y, Seki A, Sumiyoshi T, Matsui M, Goto T, Tanabe Y, Sueda S, Sato T, Ogawa S, Kubo N, Momomura SI, Ogawa H, Shimokawa H. Clinical implications of provocation tests for coronary artery spasm: safety, arrhythmic complications, and prognostic impact: multicentre registry study of the Japanese Coronary Spasm Association. *Eur Heart J*. 2013;34:258–267.
20. Ohba K, Sugiyama S, Sumida H, Nozaki T, Matsubara J, Matsuzawa Y, Konishi M, Akiyama E, Kurokawa H, Maeda H, Sugamura K, Nagayoshi Y, Morihisa K, Sakamoto K, Tsujita K, Yamamoto E, Yamamuro M, Kojima S, Kaikita K, Tayama S, Hokimoto S, Matsui K, Sakamoto T, Ogawa H. Microvascular coronary artery spasm presents distinctive clinical features with endothelial dysfunction as nonobstructive coronary artery disease. *J Am Heart Assoc*. 2012;1:e002485 doi: 10.1161/JAHA.112.002485.
21. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev*. 1991;43:109–142.
22. Furchgott RF. Role of endothelium in response of vascular smooth muscle. *Circ Res*. 1983;53:557–573.
23. Bassenge E. Coronary vasomotor responses: role of endothelium and nitrovasodilators. *Cardiovasc Drugs Ther*. 1994;8:600–612.
24. Nakayama M, Yasue H, Yoshimura M, Shimasaki Y, Kugiyama K, Ogawa H, Motoyama T, Saito Y, Ogawa Y, Miyamoto Y, Nakao K. T–786>C mutation in the 50-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. *Circulation*. 1999;99:2864–2870.
25. Nakayama M, Yoshimura M, Sakamoto T, Abe K, Yamamuro M, Shono M, Suzuki S, Nishijima T, Miyamoto Y, Saito Y, Nakao K, Yasue H, Ogawa H. A -786T>C polymorphism in the endothelial nitric oxide synthase gene reduces serum nitrite/nitrate levels from the heart due to an intracoronary injection of acetylcholine. *Pharmacogenet Genomics*. 2006;16:339–345.
26. Ogawa H, Akasaka T, Hattori R, Kawashima S, Kawasuji M, Kimura K, Miwa K, Mizuno K, Mohri M, Murohara T, Node K, Okumura K, Saito S, Shimokawa H, Sueda S, Takeyama Y, Tanabe Y, Tsuchihashi K, Yamagishi M, Yoshimura M, Ibuki C, Inoue T, Kaikita K, Kawano H, Kojima S, Kosuge M, Nakayama M, Oshita A, Soejima H, Takarada S, Yasuda S, Haze K, Kishida H, Tomoike H, Yokoyama M. Guidelines for diagnosis and treatment of patients with vasospastic angina (coronary spastic angina) (JCS 2008): digest version. *Circ J*. 2010;74:1745–1762.
27. Austen WG, Edwards JE, Frye RL, Gensini GG, Gott VL, Griffith LS, McGoon DC, Murphy ML, Roe BB. A reporting system on patients evaluated for coronary artery disease. Report of the Ad Hoc Committee for Grading of Coronary Artery Disease, Council on Cardiovascular Surgery, American Heart Association. *Circulation*. 1975;51:5–40.
28. Goldberg S, Lam W, Mudge G, Green LH, Kushner F, Hirshfeld JW, Kastor JA. Coronary hemodynamic and myocardial metabolic alterations accompanying coronary spasm. *Am J Cardiol*. 1979;43:481–487.
29. Hsueh WA, Quiñones MJ. Role of endothelial dysfunction in insulin resistance. *Am J Cardiol*. 2003;92:10J–17J.
30. Balletshofer BM, Rittig K, Enderle MD, Volk A, Maerker E, Jacob S, Matthaei S, Rett K, Häring HU. Endothelial dysfunction is detectable in young normotensive first-degree relatives of subjects with type 2 diabetes in association with insulin resistance. *Circulation*. 2000;101:1780–1784.
31. Clarkson P, Celermajer DS, Donald AE, Sampson M, Sorensen KE, Adams M, Yue DK, Betteridge DJ, Deanfield JE. Impaired vascular reactivity in insulin-dependent diabetes mellitus is related to disease duration and low density lipoprotein cholesterol levels. *J Am Coll Cardiol*. 1996;28:573–579.
32. Mäkimattila S, Virkamäki A, Groop PH, Cockcroft J, Utriainen T, Fagerudd J, Yki-Järvinen H. Chronic hyperglycemia impairs endothelial function and insulin sensitivity via different mechanisms in insulin-dependent diabetes mellitus. *Circulation*. 1996;94:1276–1282.
33. Quiñones MJ, Hernandez-Pampaloni M, Schelbert H, Bulnes-Enriquez I, Jimenez X, Hernandez G, De La Rosa R, Chon Y, Yang H, Nicholas SB, Modilevsky T, Yu K, Van Herle K, Castellani LW, Elashoff R, Hsueh WA. Coronary vasomotor abnormalities in insulin-resistant individuals. *Ann Intern Med*. 2004;140:700–708.
34. Hsueh WA, Lyon CJ, Quiñones MJ. Insulin resistance and the endothelium. *Am J Med*. 2004;117:109–117.