history of myocardial infarction and stroke in the Third National Health and Nutrition Examination Survey. Circulation, 2004; 109: 42-46
19) Takeuchi H, Saitoh S, Takagi S, Ohnishi H, Ohhata J, Isobe T, Shimamoto K: Metabolic syndrome and cardiac disease in Japanese men: applicability of the concept of metabolic syndrome defined by the National Cholesterol Education Program-Adult Treatment Panel III to Japanese men--the Tanno and Sobetsu Study. Hypertens Res, 2005; 28: 203-208
20) Deedwania P, Barter P, Carmena R, Fruchart JC, Grundy SM, Haffner S, Kastelein JJ, LaRosa JC, Schachner H, Shepherd J, Waters DD; Treating to New Targets Investigators: Reduction of low-density lipoprotein cholesterol in patients with coronary heart disease and metabolic syndrome: analysis of the Treating to New Targets study. Lancet, 2006; 368: 919-928
21) Ninomiya T, Kubo M, Doi Y, Yonemoto K, Tanizaki Y, Rahman M, Arima H, Tsuryuya K, Iida M, Kiyohara Y: Impact of metabolic syndrome on the development of cardiovascular disease in a general Japanese population: the Hisayama study. Stroke, 2007; 38: 2063-2069
22) Gami AS, Witt BJ, Howard DE, Erwin PJ, Gami LA, Somers VK, Montori VM: Metabolic syndrome and risk of incident cardiovascular events and death: a systematic review and meta-analysis of longitudinal studies. J Am Coll Cardiol, 2007; 49: 403-414
23) Lutsey PL, Steffen LM, Stevens J: Dietary intake and the development of the metabolic syndrome: the Atherosclerosis Risk in Communities study. Circulation, 2008; 117: 754-761
24) Teramoto T, Sasaki J, Ueshima H, Egusa G, Kinoshita M, Shimamoto K, Daida H, Biro S, Hirobe K, Funahashi T, Yokote K, Yokode M: Metabolic syndrome. J Atheroscler Thromb, 2008; 15: 1-5
25) Ministry of Health, Labour, and Welfare of Japan: The National Health and Nutrition Survey in Japan 2005. Tokyo, Japan: Daiichi Syuppan, 2006 (In Japanese)
26) Kubota I, Ito H, Yokoyama K, Yasumura S, Tomoike H: Early mortality after acute myocardial infarction. Observation study in Yamagata, 1993-1995. Jpn Circ J, 1998; 62: 414-418
27) Kubota I, Matsui M, Ito H, Saito M, Yokoyama K, Yasumura S, Tomoike H: Long-term prognosis after recovery from myocardial infarction: a community-based survey in Yamagata, Japan. Intern Med, 2001; 40: 589-593
28) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults: Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). JAMA, 2001; 285: 2486-2497
29) Hao Z, Konta T, Takasaki S, Abiko H, Ishikawa M, Takahashi T, Ikeda A, Ichikawa K, Kawata S, Kato T, Kubota I: The association between microalbuminuria and metabolic syndrome in the general population in Japan: the Takahata study. Intern Med, 2007; 46: 341-346
30) Konta T, Hao Z, Abiko H, Ishikawa M, Takahashi T, Ikeda A, Ichikawa K, Takasaki S, Kubota I: Prevalence and risk factor analysis of microalbuminuria in Japanese general population: the Takahata study. Kidney Int, 2006; 70: 751-756
31) Sattar N, McConnachie A, Shaper AG, Blauw GJ, Buckley BM, de Craen AJ, Ford I, Forouhi NG, Freeman DJ, Jukema JW, Lennon L, Macfarlane PW, Murphy MB, Packard CJ, Stott DJ, Westendorp RG, Whincup PH, Shepherd J, Wannamethee SG: Can metabolic syndrome usefully predict cardiovascular disease and diabetes? Outcome data from two prospective studies. Lancet, 2008; 371: 1927-1935
32) Castelli WP: Epidemiology of coronary heart disease: the Framingham study. Am J Med, 1984; 76: 4-12
33) Shiraishi J, Kohno Y, Sawada T, Nishizawa S, Arihara M, Hadase M, Hyogo M, Yagi T, Shima T, Nakazawa A, Shigeta M, Yamada H, Tatsumi T, Azuma A, Matsubara H; AMI-Kyoto Multi-Center Risk Study Group: Relation of obesity to acute myocardial infarction in Japanese patients. Circ J, 2006; 70: 1525-1530
34) Maruyama K, Hirobe K, Noda H, Iso H, Dohi S, Terai T, Fujioka S, Goto K, Horie S, Nakano S: Associations between blood lipid profiles and risk of myocardial infarction among Japanese male workers: 3M Study. J Atheroscler Thromb, 2009; 16: 714-721
35) Satoh H, Tomita K, Fujii S, Kishi R, Tsutsui H: Lower high-density lipoprotein cholesterol is a significant and independent risk for coronary artery disease in Japanese men. J Atheroscler Thromb, 2009; 16: 792-798
36) Teramoto T, Sasaki J, Ueshima H, Egusa G, Kinoshita M, Shimamoto K, Daida H, Biro S, Hirobe K, Funahashi T, Yokote K, Yokode M: Risk factors of atherosclerotic diseases. Executive summary of Japan Atherosclerosis Society (JAS) guideline for diagnosis and prevention of atherosclerosis cardiovascular diseases for Japanese. J Atheroscler Thromb, 2007; 14: 267-277
37) Anand SS, Islam S, Rosengren A, Franzosi MG, Steyn K, Yusufali AH, Keltai M, Diaz R, Rangarajan S, Yusuf S; INTERHEART Investigators: Risk factors for myocardial infarction in women and men: insights from the INTERHEART study. Eur Heart J, 2008; 29: 932-940
38) Kannel WB, Wilson PW: Risk factors that attenuate the female coronary disease advantage. Arch Intern Med, 1995; 155: 57-61
39) Stampfer MJ, Colditz GA, Willett WC, Manson JE, Rosner B, Speizer FE, Hennekens CH: Postmenopausal estrogen therapy and cardiovascular disease. Ten-year follow-up from the nurses' health study. N Engl J Med, 1991; 325: 756-762

Heart Vessels (2008) 23:83–90
DOI 10.1007/s00380-007-1019-4

ORIGINAL ARTICLE

**Yuichi Ninomiya · Shuichi Hamasaki · Keishi Saihara
Sanemasa Ishida · Tetsuro Kataoka · Masakazu Ogawa
Koji Orihara · Naoya Oketani · Tsuyoshi Fukudome
Hideki Okui · Tomoko Ichiki · Takuro Shinsato
Takuro Kubozono · Etsuko Mizoguchi · Hitoshi Ichiki
Chuwa Tei**

Comparison of effect between nitrates and calcium channel antagonist on vascular function in patients with normal or mildly diseased coronary arteries

Received: December 22, 2006 / Accepted: September 21, 2007

Abstract The comparative long-term antianginal efficacy of long-acting nitrates versus calcium channel antagonists remains unclear. The goal of the present study was to compare the coronary endothelial cell function and coronary artery vasoconstriction between patients with normal or mildly diseased coronary arteries treated with long-acting nitrates or calcium channel antagonists. Forty-two patients suspected to have angina pectoris and with normal or mildly diseased coronary arteries underwent Doppler flow study of the left anterior descending coronary artery. All patients were suspected to have angina pectoris and were receiving either long-acting nitrates ($n = 18$; Nitrates group) or calcium channel antagonists ($n = 24$; Ca-antagonists group) for at least 1 year. Vascular reactivity was assessed by intracoronary administration of papaverine, acetylcholine (Ach), and nitroglycerin using a Doppler guidewire. Segments that showed the greatest constrictive response to Ach were used for assessment of vasoconstriction. The percent increase in coronary blood flow (CBF) and coronary artery diameter (CAD) induced by Ach was significantly smaller in the Nitrates group than in the Ca-antagonists group (33% ± 74% vs 83% ± 77%, $P < 0.05$; –3% ± 16% vs 11% ± 12%, $P < 0.01$, respectively). The percent diameter reduction in the region of greatest constrictive response to Ach was significantly greater in the Nitrates group than in the Ca-antagonists group (44% ± 39% vs 15% ± 32%, $P < 0.02$). Long-term treatment with long-acting nitrates may produce less favorable effects on coronary endothelial function and the constrictive response to Ach when compared with long-acting calcium channel antagonists in patients with normal or mildly diseased coronary arteries.

Key words Nitrates · Endothelial function · Shear stress · Vasoconstriction

Y. Ninomiya[1] · S. Hamasaki[1] (✉) · K. Saihara[1] · S. Ishida · T. Kataoka · M. Ogawa · K. Orihara · N. Oketani · T. Fukudome · H. Okui · T. Ichiki · T. Shinsato · T. Kubozono · E. Mizoguchi · H. Ichiki · C. Tei
Department of Cardiovascular, Respiratory and Metabolic Medicine, Graduate School of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan
Tel. +81-99-275-5318; Fax +81-99-265-8447
e-mail: hamasksh@m.kufm.kagoshima-u.ac.jp

[1]These authors contributed equally to this study.

Introduction

Nitrates are widely used for the treatment of angina pectoris, and their ability to reduce left ventricular remodeling and cardiac mortality during acute myocardial infarction has been well described.[1,2] Recent large mega-trials (GISSI-3 and ISIS-4) failed to demonstrate any benefit for nitroglycerin on mortality in patients with acute myocardial infarction. Since these studies utilized populations that received nitrates for 5–6 weeks, the effect of long-term administration of nitrates remains unclear.[3,4] However, Ishikawa et al.[5] suggested that long-term use of nitrates increased cardiac events in patients with previous myocardial infarction. The pharmacologic and physiologic mechanisms of this unfavorable result remain unknown, but may involve neurohormonal counter-regulatory mechanisms, impaired nitrates biotransformation, or intrinsic changes in the vasculature.[6–8]

Calcium (Ca) channel antagonists have also been used for the treatment of angina pectoris. The negative cardiovascular impact of some short-acting formulations of Ca channel antagonists was described in the 1990s and has led to the reduced use of Ca channel antagonists.[9–11] However, several recent randomized studies have suggested that long-acting Ca channel antagonists are safe and beneficial for the treatment of coronary artery disease.[12–14]

Many patients were suspected to have angina pectoris based on clinical history of chest pain symptoms elicited by their physicians rather than by objective findings on cardiac catheterization, and these patients were treated with either nitrates or calcium channel antagonists. Many studies have demonstrated that endothelial dysfunction is one of the earliest markers in patients with atherogenic risk factors

(e.g., male gender, aging, hypertension, diabetes mellitus, smoking, family history) in the absence of angiographic evidence of atherosclerosis.[15-17] Further, statins, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, and long-acting Ca-antagonists have been reported to alleviate endothelial dysfunction.[18,19] Therefore, patients with normal or mildly diseased coronary arteries were selected as the study population with the goal of comparing coronary endothelial cell function and coronary artery vasoconstriction between the patients treated with long-acting nitrates and those treated with calcium channel antagonists. Moreover, to explore the hypothesis that shear stress may be associated with impairments in coronary endothelial function, this study investigated shear stress and endothelial function in patients undergoing treatment with long-acting nitrates or calcium channel antagonists.

Subjects and methods

Study population

Forty-two patients with suspected angina pectoris (15 women, 27 men; 63 ± 12 years) and normal coronary caliber or mildly stenotic coronary lesions (% diameter stenosis <30%) were enrolled in this study. All participants were receiving long-acting nitrates (Nitrates groups) or calcium channel antagonists (Ca-antagonists group) and underwent Doppler flow study of the left anterior descending coronary artery. The Nitrates group consisted of 18 patients undergoing nitrate therapy for at least 1 year (average of 2.5 years), and the Ca-antagonists group consisted of 24 patients undergoing treatment with Ca channel antagonists for at least 1 year (average of 2.3 years). Inclusion criteria were: (1) angiographically smooth arteries; (2) mild irregularities with no coronary artery lesion >30% lumen diameter stenosis by visual assessment in major epicardial vessel; and (3) proximal coronary arteries >2.0 mm. Patients with previous myocardial infarction, previous coronary revascularization, valvular heart disease, vasospastic angina, cardiomyopathy, or myocarditis were excluded from study. Nitrates or Ca channel antagonists were administered orally during the study, in continuous dosing; patients in the Nitrates group received long-acting isosorbide dinitrate (40 mg/day) or long-acting isosorbide mononitrate (40 mg/day), while patients in Ca-antagonists group received long-acting amlodipine besylate (5 mg/day), or long-acting nifedipine (20 mg/day). Written informed consent was obtained from all patients before catheterization in accordance with guidelines established by the Committee for the Protection of Human Subjects in our institution.

Study protocol

Diagnostic coronary angiography was performed using a 6-F Judkins catheter with a standard femoral percutaneous approach. Five thousand units of heparin were administered at the beginning of the procedure. Nonionic contrast material was used for all patients. No nitroglycerin was given prior to the diagnostic procedure.

Coronary blood flow response to papaverine, acetylcholine (Ach), and nitroglycerin was studied according to previous reports.[20-22] After completion of the diagnostic catheterization, interventions were performed as follows: (1) a 0.014-inch Doppler guidewire (Cardiometrics, Santa Anna, CA, USA) was introduced into the left anterior descending coronary artery; (2) after obtaining a stable Doppler signal, a bolus of papaverine (an endothelium-independent vasodilator in resistance coronary arteries) (12.5 mg/5 ml) was injected through a catheter; (3) infusion of Ach (an endothelium-dependent vasodilator in resistance and epicardial coronary arteries) (0.5 ml/min) at dosages of either 3 or 30 μg/min for 2 min was performed via the catheter;[23,24] and (4) a bolus of nitroglycerin (an endothelium-independent vasodilator in epicardial coronary arteries) (200 μg/5 ml) was administered. Drugs were infused at least 5 min apart. Coronary angiography was performed before and 2 min after each dose of Ach and after administration of nitroglycerin. The infusion of Ach was terminated either when a significant vessel constriction occurred or when the dose of 30 μg/min was reached. Phasic coronary blood flow velocities, arterial blood pressure, and heart rate were monitored continuously and recorded. Measurements obtained during steady state conditions were used as control values for later analysis.

Quantitative coronary angiographic images

Technically suitable single-plane angiograms were selected for computer analysis. Quantitative coronary angiographic images (DBAC-1000; MID, Fukuoka, Japan) were recorded using validated densitometric analysis, as previously reported.[25] Endothelium-dependent and -independent vasodilation of the epicardial coronary artery was estimated by measuring the coronary artery luminal diameter at the tip of the Doppler guidewire. Segments showing the greatest constrictive response to Ach in the left anterior descending coronary artery were used for analysis of vasoconstriction. The degree of vasoconstriction induced by Ach was normalized by the diameter obtained at baseline and is presented as the percent diameter reduction.

Assessment of coronary blood flow

Doppler flow velocity spectra were analyzed on-line to determine time-averaged peak velocity. Volumetric coronary blood flow (CBF) was determined using the formula: CBF = cross-sectional area × average peak velocity × 0.5.[26] Coronary flow reserve (CFR) to papaverine was calculated as the ratio of maximal CBF induced by papaverine to basal CBF, which reflects the endothelium-independent function of the resistance coronary artery. Endothelium-dependent function was calculated as the percent increase of CBF or coronary artery diameter (CAD) in response to Ach. Endothelium-independent vasodilation of the epicardial

coronary artery was assessed by the percent increase of CAD in response to nitroglycerin.[20–22]

Coronary wall shear stress

The Doppler guidewire tip was placed at the target lesion to record average peak velocity at baseline. All parameters were calculated on a beat-to-beat basis for 30s and averaged. Aortic blood was then collected to measure blood viscosity. Coronary wall shear stress (in dynes per centimeter squared) was estimated by the Hägen–Poiseuille formula $(4\mu Q)/(\pi r^3)$, where Q is coronary blood flow, μ is the blood viscosity, and r is the radius of the lumen. Dynamic viscosity, μ, was calculated from the shear rate, hematocrit, and total protein, according to the regression equations described by de Simone et al.[27]

Statistical analysis

Values are expressed as the mean ± SD. Statistical significance was accepted when the P value was less than 0.05. The relationship between two parameters was evaluated with a linear regression analysis. Comparison of the baseline cardiovascular risk variables between the two groups was performed using Pearson's chi-square test, and comparisons of hemodynamic and echocardiographic data between the study groups were performed using one-way analysis of variance.

Results

A total of 42 patients were evaluated. Patient characteristics of both groups are summarized in Table 1. Gender distribution, age, body mass index (BMI), New York Heart Association (NYHA) classification, and additional cardiovascular drugs were similar when comparing the two groups. The prevalence of hypertension and smoking were significantly higher in the Ca-antagonists group than in the Nitrates group, whereas the prevalence of hyperlipidemia and diabetes were similar when comparing the two groups. Total-cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, fasting plasma sugar, left ventricular dimension at diastole and systole, intraventricular septum thickness, left ventricular (LV) posterior wall thickness, LV mass, and systolic, diastolic, and mean arterial pressure were also similar when comparing the two groups (Table 2).

Coronary wall shear stress

Comparisons of coronary wall shear stress are shown in Fig. 1. Coronary wall shear stress at baseline was significantly greater in the Nitrates group than in the Ca-antagonists group (109 ± 54 vs 76 ± 30 dynes/cm^2, $P < 0.02$). Both coronary artery diameter (CAD) and coronary blood flow (CBF) at baseline were similar when comparing the Nitrates group and the Ca-antagonists group (2.9 ± 0.4 vs 3.2 ± 0.6mm; 88 ± 46 vs 83 ± 58ml/min, respectively).

Changes in coronary blood flow

The percent change in CBF induced by papaverine and Ach is shown in Fig. 2. The percent increase in CBF induced by Ach was significantly smaller in the Nitrates group than in the Ca-antagonists group (33% ± 74% vs 83% ± 77%, $P < 0.05$). The percent change in CBF induced by papaverine tended to be smaller in the Nitrates group than in the Ca-antagonists group (195% ± 93% vs 230% ± 94%), but this difference did not reach the level of statistical significance.

Table 1. Patient characteristics

	Nitrates group ($n = 18$)	Ca-antagonists group ($n = 24$)	
Men	10/18 (56%)	17/24 (71%)	NS
Age (years)	63 ± 17	63 ± 8	NS
BMI (kg/m^2)	24.0 ± 4.1	24.0 ± 3.5	NS
Hyperlipidemia	7/18 (39%)	8/24 (33%)	NS
Diabetes	4/18 (22%)	5/24 (21%)	NS
Hypertension	7/18 (39%)	19/24 (79%)	$P < 0.01$
Smoking	3/18 (17%)	11/24 (46%)	$P < 0.05$
NYHA classification			
Class I	7/18 (39%)	14/24 (58%)	NS
Class II	11/18 (61%)	10/24 (42%)	NS
Medication			
ACE inhibitor	4/18 (22%)	8/24 (33%)	NS
AT-II antagonist	7/18 (39%)	7/24 (29%)	NS
β-blocker	3/18 (17%)	3/24 (13%)	NS
Nicorandil	1/18 (6%)	3/24 (13%)	NS
Statin	2/18 (11%)	3/24 (13%)	NS
Aspirin	8/18 (44%)	9/24 (38%)	NS

Values are mean ± SD
BMI, body mass index: ACE, angiotensin converting enzyme; AT-II, angiotensin II; NS, not significant

Table 2. Patient characteristics

	Nitrates group ($n = 18$)	Ca-antagonists group ($n = 24$)	
Laboratory data			
Total cholesterol (mg/dl)	187 ± 32	186 ± 32	NS
Triglyceride (mg/dl)	119 ± 45	108 ± 39	NS
HDL-cholesterol (mg/dl)	48 ± 14	53 ± 12	NS
LDL-cholesterol (mg/dl)	116 ± 26	115 ± 24	NS
Fast plasma sugar (mg/dl)	99 ± 23	105 ± 18	NS
Echocardiographic data			
LVDd (mm)	52 ± 9	52 ± 9	NS
LVDs (nm)	37 ± 13	34 ± 10	NS
IVS (mm)	14 ± 7	14 ± 6	NS
LVPW (mm)	11 ± 2	11 ± 4	NS
LVMI (g)	265 ± 92	260 ± 109	NS
Hemodynamics data			
Systolic BP (mmHg)	122 ± 20	132 ± 17	NS
Diastolic BP (mmHg)	73 ± 14	80 ± 12	NS
Mean BP (mmHg)	89 ± 16	98 ± 12	NS

Values are mean ± SD
HDL and LDL, high- and low-density lipoprotein cholesterol; LVDd, left ventricular dimension at diastole; LVDs, left ventricular dimension at systole; IVS, interventricular septum thickness; LVPW, left ventricular post wall thickness: LVMI, left ventricular mass index; BP, blood pressure; NS, not significant

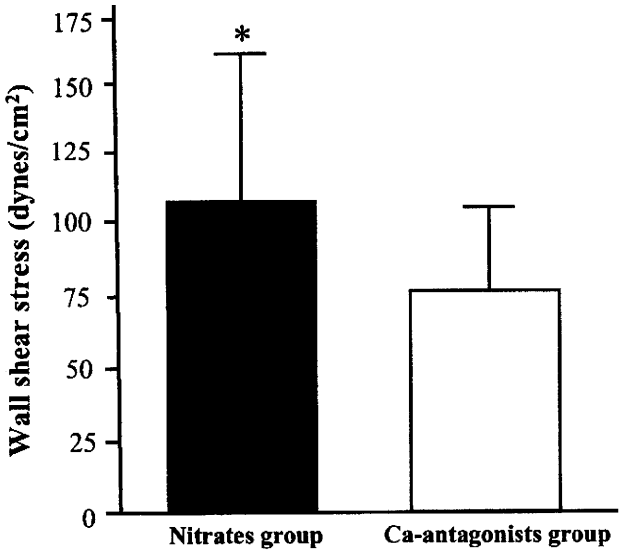

Fig. 1. Wall shear stress in the two study groups. Mean ± SD. *$P < 0.05$ vs Ca-antagonists group

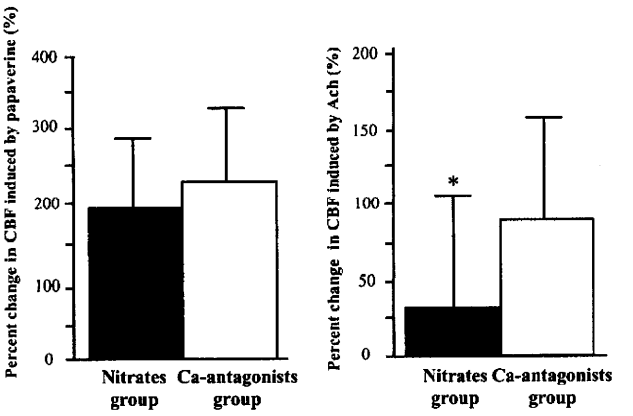

Fig. 2. Percent change in coronary blood flow (*CBF*) induced by papaverine and the percent change in CBF induced by acetylcholine (*Ach*) in the two study groups. Mean ± SD. *$P < 0.05$ vs Ca-antagonists group

Changes in coronary artery diameter

The percent change of CAD induced by Ach and nitroglycerin is shown in Fig. 3. Baseline CAD did not differ when comparing the two groups. The percent change in CAD induced by Ach was significantly smaller in the Nitrates group than in the Ca-antagonists group (−3% ± 16% vs 11% ± 12%, $P < 0.01$). The percent change in CAD induced by nitroglycerin tended to be smaller in the Nitrates group than in the Ca-antagonists group (15% ± 13% vs 21% ± 22%), but this difference did not reach the level of statistical significance.

Coronary vasoconstriction induced by Ach

Constrictor responses to Ach were quantified as percent reduction in luminal diameter relative to the luminal diameter obtained at baseline (Fig. 4). The percent reduction in luminal diameter was significantly greater in the Nitrates group than in the Ca-antagonists group (44% ± 39% vs 15% ± 32%, $P < 0.05$).

Discussion

This study may show that endothelium-dependent vasodilation of the resistance and epicardial coronary arteries are more impaired and that epicardial coronary arteries are

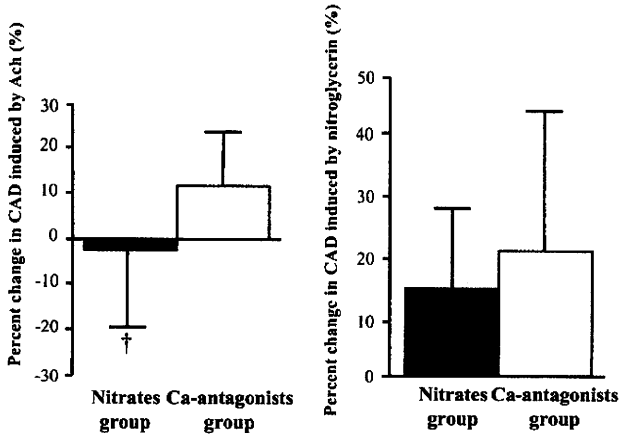

Fig. 3. Percent change in coronary artery diameter (*CAD*) induced by Ach and the percent change in CAD induced by nitroglycerin in the two study groups. Mean ± SD. †$P < 0.01$ vs Ca-antagonists group

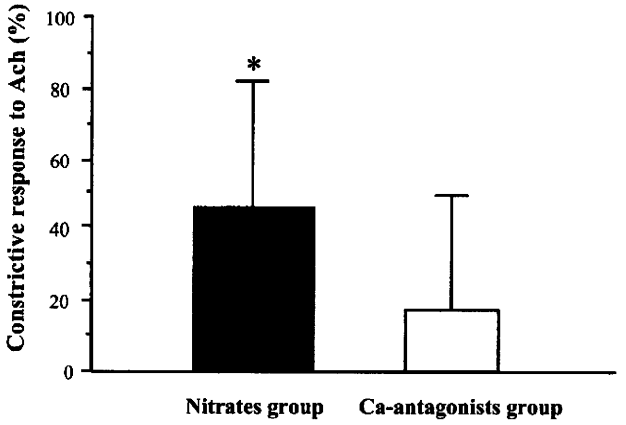

Fig. 4. Constrictive response to Ach in the two study groups. Mean ± SD. *$P < 0.05$ vs Ca-antagonists group

more vulnerable to Ach-induced coronary spasm in patients undergoing long-term treatment with nitrates when compared with those undergoing long-term treatment with Ca channel antagonists. This study may also demonstrate that long-term treatment with nitrates result in impaired endothelium-dependent coronary artery vasodilation, possibly via increases in wall shear stress.

Effect of long-term nitrate therapy

Recently, the GISSI-3 study failed to demonstrate any beneficial effect of 6 weeks of transdermal nitroglycerin therapy on mortality rate following acute myocardial infarction, and the ISIS-4 study failed to demonstrate any survival benefit in the first 5 weeks following a 1-month course of nitrate treatment.[3,4] Further, Ishikawa et al.[5] suggested that long-term treatment with nitrates increased cardiac events in patients with previous myocardial infarction. Nakamura et al.[28] performed prospective analysis of data acquired in a large, observational study involving 1042 patients enrolled in the MSMI study and 1779 patients enrolled in the MDPIT, and demonstrated that nitrate therapy was associated with a significantly increased risk of mortality in patients that had recovered from an acute coronary event.[29,30] However, these reports were different from our study because most of their subjects were patients with previous myocardial infarction who received short-term nitrates treatment. Caramori et al.[31] demonstrated exaggerated coronary vasomotor response after nitrate treatment and also described in detail impaired endothelial dysfunction elicited by long-term nitrate therapy. However, the period of nitrate therapy in that study was only 5 days, and nitrates were administered continuously via transdermal patch. In our study, patients with suspected angina pectoris received oral nitrate treatment for at least 1 year (average of 2.3 years). Furthermore, Parker and Gori[32] described in detail impaired endothelial dysfunction elicited by long-term nitrates treatment and suggested caution with this regimen.

Potential mechanisms underlying nitrate therapy and poor outcomes

Mechanisms that may account for the unfavorable effect of nitrate therapy on long-term outcomes in patients with coronary artery disease include nitrate tolerance and activation of the neurohumoral system.[33–35] Endothelial nitric oxide synthase (eNOS) is constitutively expressed and activated by cell surface receptors or mechanical forces such as shear stress and stretch.[36] Nitric oxide released by vascular endothelial cells mediates relaxation of vascular smooth muscle, inhibition of platelet activation, and modulation of migration and growth of vascular smooth muscle. Several reports suggest that alterations in the NO pathway might be involved in endothelial dysfunction and atherosclerosis.[37–40]

Shear stress is the principal fluid-mechanical signal that regulates flow-mediated remodeling and therefore provides functional assessment of the adequacy of flow-mediated remodeling.[41,42] Shear stress-mediated signal transduction is endothelium-dependent, and, in the present study, wall shear stress at baseline was greater in the Nitrates group than in the Ca-antagonists group.[43,44] Further, intracoronary Ach-induced endothelium-dependent vasodilation in conduit and resistance coronary arteries was lower in the Nitrates group than in the Ca-antagonists group.

Nitric oxide production can be stimulated by mechanical forces, such as shear stress, or by signal transduction pathways activated by Ach.[45] In the present study, the Nitrates group demonstrated impaired response to Ach and a greater response to shear stress when compared with the Ca-antagonists group. Thus, it is possible that NO overproduction in response to shear stress results in downregulation of Ach-activated signal transduction elements. Indeed, Griscavage et al.[46] reported that NO inhibits the activity of nitric oxide synthase. Therefore, NO generated from exogenous nitrate therapy may also suppress Ach-induced synthesis of NO. In the present study, shear stress was greater in the Nitrates

group than in the Ca-antagonists group. Thus, suppression of Ach-induced NO synthesis may result in less Ach-induced vasodilation in the Nitrates group than in the Ca-antagonists group.

Association between coronary endothelial function and coronary events

In this study, the prevalence of atherogenic risk factors (e.g., hypertension and smoking) were significantly higher in the Ca-antagonists group than in the Nitrates group. However, long-term treatment with long-acting nitrates was more closely associated with impaired endothelium-dependent vasodilation. Thus, the higher prevalence of hypertension and smoking in the Ca-antagonists group did not affect the results in this study.

Since the endothelium is regarded as an atheroprotective cell line, loss of endothelial cell function would logically be associated with progression of atherosclerotic disease. Indeed, Suwaidi et al.[47] reported that patients with mild coronary artery disease and severe endothelial dysfunction are at increased risk for cardiac events, and Schachinger et al.[48] demonstrated that coronary endothelial dysfunction predicted long-term atherosclerotic disease progression and cardiovascular events. These patient population of this study comprised patients without coronary artery disease. Therefore, our study suggests that treatment of patients with normal or mildly diseased coronary arteries with long-acting nitrates may be associated with cardiovascular events due to coronary endothelial dysfunction.

Abnormal vascular responses to acetylcholine, as demonstrated in the present study, may represent a reduction in NO bioavailability.[49] A decrease in NO bioavailability is associated with accelerated atherogenesis and increased monocyte–endothelial cell adhesion, which may result in local inflammation of the vascular wall and promote plaque rupture.[50,51] Further, absence of an appropriate increase in blood flow secondary to endothelial cell dysfunction may lead to relative myocardial ischemia even in the absence of coronary artery disease.[22,52]

Limitations

This study possesses several limitations. First, the calculations used for shear stress assumed a steady laminar flow in a circular, rigid, nontapering tube. Therefore, the current study investigated hemodynamic in circular, discrete, and mildly stenotic target lesions while excluding marked curvatures and bifurcations of the coronary arteries when measuring coronary blood flow and wall shear stress. Second, this study was not performed in a prospective and randomized fashion. A randomized, placebo-controlled trial would be of benefit in confirming the unfavorable effects of long-term nitrate therapy on coronary endothelial function and coronary vasoconstriction. Third, only patients with normal or mildly diseased coronaries were studied. Thus, the findings of this study cannot be applicable to the patients with significant coronary artery disease. Finally, the response to papaverine, acetylcholine, and nitroglycerin was tested without withdrawal of nitrates or calcium antagonists. However, we performed coronary catheterization in combination with a Doppler flow study without stopping these medications for the following reasons. First, the prevalence of coronary spastic angina is apparently higher in Japanese patients with coronary heart disease than in their Caucasian counterparts.[53] Thus, we were afraid that if we stopped these medications, a coronary spasm would occur during control coronary angiography and disturb the exact estimation of coronary function. Second, we were interested in the direct effect of nitrates and calcium channel antagonists on coronary endothelial function and coronary artery vasoconstriction at the time when the patients were under the effect of these medications.

Conclusions

When compared with long-term treatment with long-acting Ca channel antagonists, long-term treatment with long-acting nitrates may be more closely associated with impaired endothelium-dependent vasodilation in conduit and resistance coronary arteries and with an exaggerated vasoconstrictive response in conduit coronary arteries in patients with normal or mildly diseased coronary arteries, potentially via increases in wall shear stress.

References

1. Yusuf S, Collins R, McMahon S, Peto R (1988) Effect of intravenous nitrates on mortality in acute myocardial infarction: An overview of the randomized trials. Lancet 1:1088–1092
2. Jugdutt BI (1992) Role of nitrates after acute myocardial infraction. Am J Cardiol 70:82B–87B
3. GISSI-3 (1994) Effects of lisinopril and transdermal glyceryl trinitrate singly and together on 6-week mortality and ventricular function after acute myocardial infarction. Gruppo Italiano per lo Studio della Sopravvivenza nell'infarto Miocardico. Lancet 343:1115–1122
4. ISIS-4 (1995) A randomized factorial trial assessing early oral captopril, oral mononitrate, and intravenous magnesium sulphate in 58,050 patients with suspected acute myocardial infarction. Lancet 345:669–685
5. Ishikawa K, Kanamasa K, Ogawa I, Takenaka T, Naito T, Kamata N, Yamamoto T, Nakai S, Hama J, Oyaizu M, Kimura A, Yamamoto K, Aso N, Arai M, Yabushita H, Katori R (1996) Long-term nitrate treatment increases cardiac events in patients with healed myocardial infarction. Jpn Circ J 60:779–788
6. Munzel T, Giaid A, Kurz S, Stewart DJ, Harrison DG. (1995) Evidence for a role of endothelin 1 and protein kinase C in nitroglycerin tolerance. Proc Natl Acad Sci USA 92:5244–5248
7. Zierhut W, Ball HA (1996) Prevention of vascular nitroglycerin tolerance by inhibition of protein kinase C. Br J Pharmacol 119:3–5
8. Heitzer T, Just H, Meinertz T, Brockhoff C, Munzel T (1998) Chronic angiotensin converting enzyme inhibition with captopril prevents nitroglycerin induced hypersensitivity to vasoconstrictors in patients with stable coronary artery disease. J Am Coll Cardiol 31:83–88
9. Furberg CD, Psaty BM, Meyer JV (1995) Nifedipine: dose-related increase in mortality in patients with coronary heart disease. Circulation 92:1326–1331

10. Thadani U, Glasser S, Bittar N, Beach CI (1994) Dose-response evaluation of once-daily therapy with a new formation of diltiazem for stable angina pectoris. Diltiazem CD Study Group. Am J Cardiol 74:9–17
11. Gibbs JS, McAlpine HM, Wright C, McLenachan JM, Sparrow J, Sutton G, Dargie HJ, Fox KM (1991) Double-blind randomized placebo-controlled dose-efficacy study of sustained release verapamil in chronic stable angina. Int J Cardiol 31:281–286
12. Poole-Wilson PA, Lubsen J, Kirwan BA, van Dalen FJ, Wagener G, Danchin N, Just H, Fox KA, Pocock SJ, Clayton TC, Motro M, Parker JD, Bourassa MG, Dart AM, Hildebrandt P, Hjalmarson A, Kragten JA, Molhoek GP, Otterstad JE, Seabra-Gomes R, Soler-Soler J, Weber S; A Coronary disease Trial Investigating Outcome with Nifedipine gastrointestinal therapeutic system investigators (2004) Effect of long-acting nifedipine on mortality and cardiovascular morbidity in patients with stable angina requiring treatment (ACTION trial): randomized controlled trial. Lancet 364:849–857
13. Nissen SE, Tuzcu EM, Libby P, Thompson PD, Ghali M, Garza D, Berman L, Shi H, Buebendorf E, Topol EJ; CAMELOT Investigators (2004) Effect of antihypertensive agents on cardiovascular events in patients with coronary disease and normal blood pressure: the CAMELOT study: a randomized controlled trial. JAMA 292:2217–2225
14. Pitt B, Byington RP, Furberg CD, Hunninghake DB, Mancini GB, Miller ME, Riley W (2000) Effect of amlodipine on the progression of atherosclerosis and the occurrence of clinical events. PREVENT Investigators. Circulation 102:1503–1510
15. Egashira K (2002) Clinical importance of endothelial function in arteriosclerosis and ischemic heart disease. Circ J 66(6):529–533
16. Egashira K, Suzuki S, Hirooka Y, Kai H, Sugimachi M, Imaizumi T, Takeshita A (1995) Impaired endothelium-dependent vasodilation of large epicardial and resistance coronary arteries in patients with essential hypertension. Different responses to acetylcholine and substance P. Hypertension 25(2):201–206
17. Zeiher AM, Schachinger V, Minners J (1995) Long-term cigarette smoking impairs endothelium-dependent coronary arterial vasodilator function. Circulation 92(5):1094–1100
18. Matsuo T, Iwade K, Hirata N, Yamashita M, Ikegami H, Tanaka N, Aosaki M, Kasanuki H (2005) Improvement of atrial stiffness by the antioxidant and anti-inflammatory effects of short-term statin therapy in patients with hypercholesterolemia. Heart Vessels 20(1):8–12
19. Takagi A, Tsurumi Y, Ishizuka N, Omori H, Arai K, Hagiwara N, Kasanuki H (2006) Single administration of cerivastatin, an HMD-CoA reductase inhibitor, improves the coronary flow velocity reserve: a transthoracic Doppler echocardiography study. Heart Vessels 21(5):298–301
20. Nishimura RA, Lerman A, Chesebro JH, Ilstrup DM, Hodge DO, Higano ST, Holmes DR Jr, Tajik AJ (1995) Epicardial vasomotor responses to acetylcholine are not predicted by coronary atherosclerosis as assessed by intracoronary ultrasound. J Am Coll Cardiol 26:41–49
21. Lerman A, Holmes DR Jr., Bell MR, Garratt KN, Nishimura RA, Burnett JC Jr (1995) Endothelin in coronary endothelial dysfunction and early atherosclerosis in humans. Circulation 92:2426–2431
22. Hasdai D, Gibbons RJ, Holmes DR Jr., Higano ST, Lerman A (1997) Coronary endothelial dysfunction in humans is associated with myocardial perfusion defects. Circulation 96:3390–3395
23. Egashira K, Inou T, Hirooka Y, Kai H, Sugimachi M, Suzuki S, Kuga T, Urabe Y, Takeshita A (1993) Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. Circulation 88:77–81
24. Fukuda Y, Teragawa H, Matsuda K, Yamagata T, Matsuura H, Chayama-K (2002) Tetrahydrobiopterin restores endothelial function of coronary arteries in patients with hypercholesterolemia. Heart 87:264–269
25. Ishida S, Hamasaki S, Kamekou M, Yoshitama T, Nakano F, Yoshikawa A, Kataoka T, Saihara K, Minagoe S, Tei C (2003) Advancing age is associated with diminished vascular remodeling and impaired vasodilation in resistance coronary arteries. Coronary Artery Dis 14:443–449
26. Doucette JW, Corl PD, Payne HM, Flynn AE, Goto M, Nassi M, Segal J (1992) Validation of a Doppler guide wire for intravascular measurement of coronary artery flow velocity. Circulation 85: 1899–1911
27. de Simone G, Devereux RB, Chien S, Alderman MH, Atlas SA, Laragh JH (1990) Relation of blood viscosity to demographic and physiologic variables and to cardiovascular risk factors in apparently normal adults. Circulation 81:107–117
28. Nakamura Y, Moss AJ, Brown MW, Kinoshita M, Kawai C (1999) Long-term nitrate use may be deleterious in ischemic heart disease: A study using the databases from two large-scale postinfarction studies. Am Heart J 138:577–585
29. Moss AJ, Goldstein RE, Hall WJ, Bigger JT Jr., Fleiss JL, Greenberg H, Bodenheimer M, Krone RJ, Marcus FI, Wackers FJ, Benhorin J, Brown MW, Case R, Coromilas J, Dwyer EM Jr., Gillespie JA, Gregory JJ, Kleiger R, Lichstein E, Parker JO, Raubertas RF, Stern S, Tzivoni D, Voorhees LV, for the Multicenter Myocardial Ischemia Group (1993) Detection and significance of myocardial ischemia in stable patients after recovery from an acute coronary event. Multicenter Myocardial Ischemia Research Group. JAMA 269:2379–2385
30. The Multicenter Diltiazem Post-infarction Trial Group (1988) The effect of diltiazem on mortality and reinfarction after myocardial infraction. N Engl J Med 319:385–392
31. Caramori PR, Adelman AG, Azevedo ER, Newton GE, Parker AB, Parker JD (1998) Therapy with nitroglycerin increases coronary vasoconstriction in response to acetylcholine. J Am Coll Cardiol 32(7):1969–1974
32. Parker JD, Gori T (2001) Tolerance to the organic nitrates: new ideas, new mechanisms, continued mystery. Circulation 104(19): 2263–2265
33. Mangione NJ, Glasser SP (1994) Phenomenon of nitrate tolerance. Am Heart J 128:137–146
34. Dupuis J, Lalonde G, Lemieux R, Rouleau JL (1990) Tolerance to intravenous nitroglycerin in patients with congestive heart failure: role of increased intravascular volume, neurohumoral activation and lack of prevention with N-Acetylcysteine. J Am Coll Cardiol 16:923–931
35. Keller LH, Hulley SB, Cohen JD, Neaton J (1986) Unexpected effects of treating hypertension in men with electrocardiographic abnormalities: a critical analysis. Circulation 73:114–123
36. Topper JN, Cai J, Falb D, Gimbrone MAJ (1996) Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. Proc Natl Acad Sci USA 93:10417–10422
37. Feron O, Dessy C, Moniotte S, Desager JP, Balligand JL (1999) Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase. J Clin Invest 103:897–905
38. Chou TC, Yen MH, Li CY, Ding YA (1998) Alterations of nitric oxide synthase expression with aging and hypertension in rats. Hypertension 31:643–648
39. Kawano H, Motoyama T, Hirashima O, Hirai N, Miyao Y, Sakamoto T, Kugiyama K, Ogawa H, Yasue H (1999) Hyperglycemia rapidly suppresses flow-mediated endothelium-dependent vasodilation of brachial artery. J Am Coll Cardiol 34:146–154
40. Oemar BS, Tschudi MR, Godoy N, Brovkovich V, Malinski T, Luscher TF (1998) Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis. Circulation 97:2494–2498
41. Davies PF (1995) Flow-mediated endothelial mechanotransduction. Physiol Rev 75:519–560
42. Gimbrone MA Jr, Topper JN, Nagel T, Anderson KR, Garcia-Cardena G (2000) Endothelial dysfunction, hemodynamic forces, and atherogenesis. Ann N Y Acad Sci 902:230–239
43. Gotlieb AI, Langille BL (1996) The role of rheology in atherosclerotic coronary artery disease. In: Fuster V, Ross R, Topol EJ (eds) Atherosclerosis and coronary artery disease. Lippincott-Raven, Philadelphia, pp 595–606
44. Ben Drias A, Benessiano J, Poitevin P, Levy BI, Michel JB (1997) Arterial expansive remodeling induced by high flow rates. Am J Physiol 272:H851–858
45. Fukaya Y, Ohhashi T (1996) Acetylcholine- and flow-induced production and release of nitric oxide in arterial and venous endothelial cells. Am J Physiol 270:H99–106

46. Griscavage JM, Fukuto JM, Komori Y, Ignarro LJ (1994) Nitric oxide inhibits neuronal nitric oxide synthase by interacting with the heme prosthetic group. Role of tetrahydrobiopterin in modulating the inhibitory action of nitric oxide. J Biol Chem 269: 21644–21649
47. Suwaidi J AL, Hamasaki S, Higano ST, Nishimura RA, Holmes DR Jr, Lerman A (2000) Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. Circulation 101:948–954
48. Schachinger V, Britten MB, Zeiher AM (2000) Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation 101:1899–1906
49. Zeiher AM, Drexeler H, Wollschlyger H, Just H (1991) Modulation of coronary vascular tone in humans: progressive endothelial dysfunction with different early stages of coronary atherosclerosis. Circulation 83:391–401
50. De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MA Jr, Shin WS, Liao JK (1995) Nitric oxide decreases cytokine-induced endothelial activation: nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. J Clin Invest 96:60–68
51. van der Wal AC, Becker AE, van der Loos CM, Das PK (1994) Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaque is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation 89:36–44
52. Zeiher AM, Krause T, Schachinger V, Minners J, Moser E (1995) Impaired endothelium-dependent vasodilation of coronary resistance vessels is associated with exercise-induced myocardial ischemia. Circulation 91:2345–2352
53. Beltrame JF, Sasayama S, Maseri A (1999) Racial heterogeneity in coronary artery vasomotor reactivity: differences between Japanese and Caucasian patients. J Am Coll Cardiol 33:1442–1452

Atherosclerosis 208 (2010) 97–103

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Tacrolimus-eluting stent inhibits neointimal hyperplasia via calcineurin/NFAT signaling in porcine coronary artery model

Narisato Hamada, Masaaki Miyata*, Hideyuki Eto, Takahiro Shirasawa, Yuichi Akasaki, Aya Nagaki, Chuwa Tei

Department of Cardiovascular, Respiratory and Metabolic Medicine, Graduate School of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

ARTICLE INFO

Article history:
Received 30 October 2008
Received in revised form 8 July 2009
Accepted 15 July 2009
Available online 28 July 2009

Keywords:
Tacrolimus-eluting stent
Calcineurin
NFAT
Smooth muscle cell
Neointimal hyperplasia

ABSTRACT

Aims: The purpose is to elucidate the mechanism by which a newly developed tacrolimus-eluting stent (TES) prevents neointimal hyperplasia after stenting.
Methods and results: The three major coronary arteries in juvenile swine were randomized to implantation of either a TES or bare metal stent (BMS). Twelve weeks after stenting, the TES showed 29% less neointimal area than the BMS. Immunohistochemical staining showed that the expression of calcineurin was up-regulated in the neointima and media after stenting, and the TES inhibited this up-regulation. Western blotting demonstrated that the expression of calcineurin, nuclear factor of activated T cell (NFAT), and interleukin-2 (IL-2) was lower with the TES than with the BMS. To confirm the effect of tacrolimus on vascular smooth muscle cells (VSMCs) and its mechanism, cultured rat VSMCs were incubated with 12.5 μM of tacrolimus (tacrolimus group) or without tacrolimus (control group). The cell number of the tacrolimus group was significantly lower than that of the control group at 48 h of incubation. Western blotting demonstrated that tacrolimus decreased the expression of calcineurin, NFATc4, and IL-2 of cultured VSMCs. We confirmed that calcineurin small-interfering RNA (siRNA) decreased cell proliferation and the expression of NFATc4 and IL-2 in cultured VSMCs compared with negative control-siRNA.
Conclusion: The newly developed TES inhibited neointimal hyperplasia after stenting via the calcineurin/NFAT/IL-2 signaling pathway, which is one of several mechanisms through which TES inhibits restenosis. Calcineurin may be an important molecular target to prevent restenosis after stenting.

1. Introduction

Percutaneous coronary intervention (PCI) is a useful procedure for the treatment of coronary stenosis. Restenosis has been called the "Achilles heel" of coronary stenting and is caused by a combination of factors, including neointimal proliferation, elastic recoil, reorganization of thrombus, remodeling, and inflammation [1,2]. Currently, a number of drug-eluting stents (DES) have been developed, including different carrier stents, coatings and drugs; and these new DES are under evaluation for their effectiveness and safety [3,4] and [5]. Sirolimus-eluting stents (SESs) and paclitaxel-eluting stents (PESs) strongly reduced the incidence of restenosis compared with bare metal stents (BMSs) [6,7]. Recently, many kinds of drug-eluting stents have been investigated in the clinical setting. Positive clinical data on drug-eluting stents come from trials examining SESs in the SIRIUS Trial [8] and PESs in TAXUS-series Trial [9]. A pooled analysis demonstrated that both SESs and PESs were associated with a marked reduction in target-lesion revascularization. However, stent thrombosis after 1-year was more common with both SESs and PESs than with BMSs [10]. A systematic autopsy study reported that both SESs and PESs caused significant delay in arterial healing characterized by persistent fibrin deposition and delayed re-endothelialization when compared with sites of BMS implantation [11,12].

To reduce the risk of in-stent restenosis and interference with the natural healing response, Kaneka Corporation (Osaka, Japan) has developed a new drug-eluting stent. The new stent is a combination of a cobalt chrome (CoCr) stent, a biodegradable polymer and a pharmaceutical agent, tacrolimus [13]. Tacrolimus is a water-insoluble macrolide cytostatic immunosuppressant with both anti-proliferative and anti-inflammatory activity [14]. It has been used clinically to prevent renal and liver transplant rejection. Tacrolimus binds to the intracellular FK-binding protein (FKBP) and forms a complex that binds to calcineurin. This binding inhibits the activation of calcineurin and disrupts the dephosphorylation of nuclear factor of activated T cell (NFAT) in T cells [15]. In addition, cell culture experiments indicated that tacrolimus inhibited the proliferation of vascular smooth muscle cells (VSMCs) [16]. However, the mechanism of the anti-proliferative effect of tacrolimus on VSMCs has not yet been clarified.

* Corresponding author. Tel.: +81 99 275 5318; fax: +81 99 265 8447.
E-mail address: **miyatam@m3.kufm.kagoshima-u.ac.jp** (M. Miyata).

0021-9150/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:**10.1016/j.atherosclerosis.2009.07.040**

The purpose of this study is to elucidate the precise mechanism by which the newly developed tacrolimus-eluting stent (TES) prevents hyperplasia after stenting, using a porcine coronary model and cultured human VSMCs.

2. Materials and methods

2.1. Stent characteristics

The stent platform of this product is the balloon-expandable stent developed by the Kaneka Corporation and is made of CoCr alloy. CoCr has been successfully used for human implants such as artificial joints and dental implants. The stent is made by cutting an alloy tube with a laser and consists of two helical coils intercrossed with two phase-different links on each turn. The stent strength in the radial direction, recoil ratio, and stent shortening (the percentage change in length between the mounted condition and the expanded condition) is a medium level compared with other stents (Additional Fig. 1A). The whole surface of the stent platform is coated with a layer of a biodegradable polymer and tacrolimus. As shown in Additional Fig. 1B and C, the surface is generally smooth and there are no cracks or peelings caused by expansion. A cross-section on transmission electron microscopy shows that the drug is uniformly distributed in the polymer layer (Additional Fig. 1D).

2.2. Animal model

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996). The protocol for the animal research was approved by a local ethics review board.

Female juvenile swine LWD with a body weight of 40–60 kg were used. Swine were pre-sedated with intramuscular injection of ketamine (5 mg/kg) and xylazine (2 mg/kg). Anesthesia was initiated by 5% isoflurane, followed by orotracheal intubation and ventilation with 1.0–3.0% isoflurane, 70% N_2O, and 30% oxygen. After surgical exposure, the right femoral artery was punctured and a 6 Fr sheath was placed. The animals then received 200 IU/kg intravenous heparin. The coronary arteries were imaged using a standard angiographic technique. Target segments were selected in the right (RCA), the left anterior descending (LAD) and the left circumflex (LCX) coronary arteries. After stent implantation, cefazolin sodium (0.5 g/day) was administered by intramuscular injection for 3 days after the surgical procedure. Oral aspirin (330 mg/day) and ticlopidine (250 mg/day) were administered starting 1-day before the procedure and continuing until sacrifice. Stents (3.5 mm in diameter and 15 mm in length) were implanted in coronary arteries with a diameter of 2.8 mm (stent-to-vessel ratio of 1.25:1). Coronary vessels were randomized to receive a bare metal stent (BMS) or a TES (two TESs in two RCAs, two TESs in two LADs, two TESs in two LCxs, two BMSs in two RCAs, two BMSs in two LADs, two BMSs in two LCxs). There is no structural difference between the TES and the BMS, or differences in the delivery/deployment of the stent. The animals were euthanized and the stented coronary arteries were harvested at 2, 4, or 12 weeks after stent implantation for immunohistochemistry, at 12 weeks for morphometry, and at 4 weeks for Western blot analysis.

2.3. Quantitative coronary angiography (QCA)

Coronary imaging was performed using a GE Healthcare OEC 9800. An experienced investigator who was blinded to the randomization assignment measured the reference diameter and the minimal luminal diameter of the stented segments at follow-up angiography. The % diameter stenosis was calculated as follows: % diameter stenosis = (reference diameter − minimal luminal diameter)/reference diameter × 100.

2.4. Morphometric measurements

For the morphometric analysis, the heart was excised, and the coronary arteries were fixed with 10% buffered formalin and embedded in resin and cut into 5 μm-thick cross-sections. Each cross-section was stained by hematoxylin-eosin (HE). After digitalizing, histomorphometric measurements were performed with Scion Image (Scion Corporation, Frederick, MD) [17]. The vessel area and neointimal area in each proximal, middle and distal stented region were measured, and the % area stenosis was calculated as follows: % area stenosis = neointimal area/vessel area × 100. The % area stenoses in the three parts (proximal, middle and distal) were averaged. Researchers were blinded to the stent type.

2.5. Immunohistochemistry

For immunohistochemistry, the stent sites were dissected into blocks, and the stent wires were carefully removed. Immunohistochemical staining was carried out on paraffin-embedded sections as described previously [18]. After deparaffinization and hydration

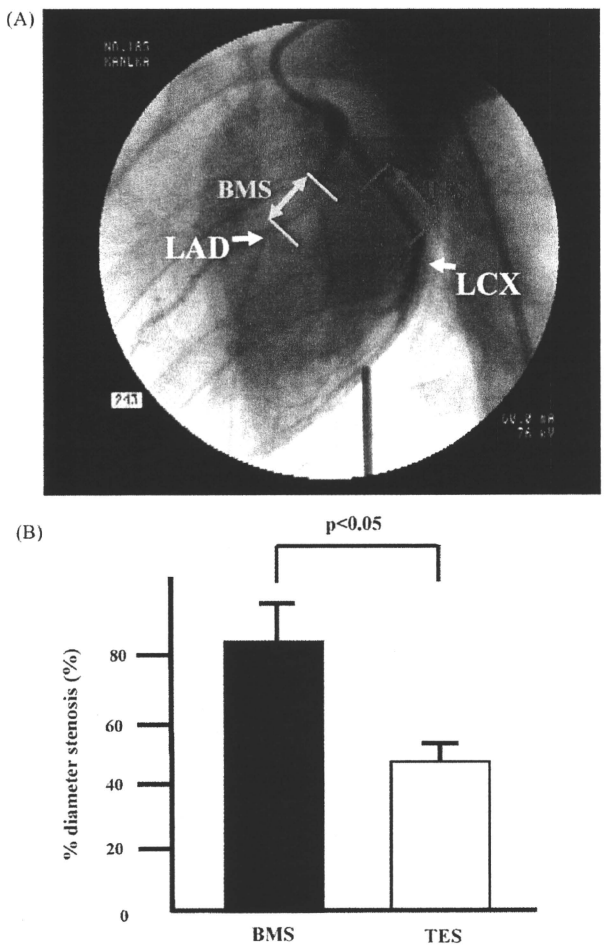

Fig. 1. Quantitative coronary angiography (QCA). (A) Representative coronary angiography 12 weeks after stent implantation shows implantation of a bare metal stent (BMS) in the left anterior descending coronary artery (LAD) and implantation of a tacrolimus-eluting stent (TES) in the left circumflex coronary artery (LCX). (B) The % diameter stenosis measured by QCA 4 weeks after stent implantation. The % diameter stenosis of the TES was significantly smaller than that of the BMS ($n = 6$).

of specimens, endogenous peroxidase activity was blocked and the specimens were fixed by immersion in 0.3% H_2O_2 in methanol for 20 min. Immunohistochemical staining was performed with a goat polyclonal antibody against human calcineurin; PP2B-Aα, von Willebrand factor (vWF), and endothelial nitric oxide synthase (eNOS) (Santa Cruz Biotechnology, Santa Cruz, CA), by the use of the labeled streptavidin biotin complex method (Simple-stain MAX-PO kit, Nichirei, Tokyo, Japan). After blocking with 10% rabbit or goat serum, slides were incubated overnight with a primary antibody at 4 °C in a moisture chamber. Slides were washed with Tris-buffered saline (TBS) and incubated with a biotinylated secondary antibody at room temperature for 30 min. After washing with TBS, slides were incubated with streptavidin at room temperature for 30 min and visualized with 3,3′-diaminobenzidine.

2.6. Western blot analysis

Protein was extracted from the neointima and media of porcine coronary arteries using the Protein and RNA Isolation System (Ambion Inc., Austin, TX). Insoluble matter was removed by centrifugation, and the protein concentration was measured by a bicinchoninic acid assay (PIERCE Biotechnology Inc., Rockford, IL). Western blotting was performed with a NuPAGE™ Electrophoresis System (Invitrogen, Carlsbad, CA) as reported previously [19]. Briefly, 10-μg protein samples were resuspended in reduced sample buffer, and then electrophoresed on a 4–12% Bis–Tris gel (Invitrogen, Carlsbad, CA) with MOPS running buffer, blotted to a nitrocellulose membrane. The protein sample was then sequentially probed with a goat polyclonal antibody against human calcineurin (PP2B-Aα), a rabbit polyclonal antibody against human NFATc4 (Santa Cruz Biotechnology), a rabbit polyclonal antibody against human interleukin-2 (IL-2) (Santa Cruz Biotechnology), and a rabbit polyclonal antibody against human actin (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated rabbit anti-goat antibody (Santa Cruz Biotechnology) or donkey anti-rabbit antibody (Santa Cruz Biotechnology) were then added, and the secondary antibody was detected by autoradiography using enhanced chemiluminescence (ECL Plus, GE Healthcare UK Ltd., Little Chalfont, UK). Densitometric analysis was performed to quantitate calcineurin, NFATc4, IL-2, and actin protein using NIH image software. Actin protein was used as a reference for quantitation of calcineurin, NFATc4, and IL-2 protein.

2.7. Cell experiments

VSMCs were isolated from the media of rat aorta. Cell culture experiments were performed as reported previously [20]. Rat VSMCs (5000) cells were seeded into 48 wells (AGC Techno Glass Co., Ltd., Chiba, Japan) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 1% fetal

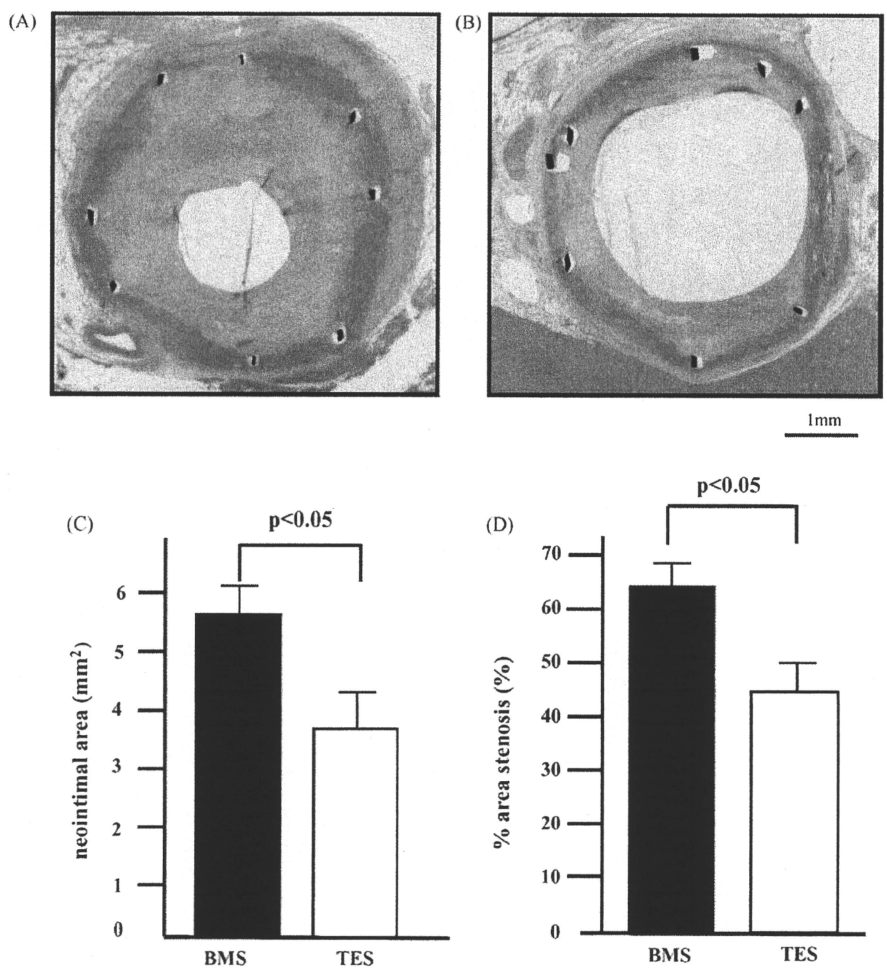

Fig. 2. Morphometric analysis at 12 weeks after stent implantation. (A and B) HE staining of cross-sections of coronary arteries implanted with a bare metal stent (BMS) (A) and a tacrolimus-eluting stent (TES) (B). (C) The neointimal area was significantly smaller with a TES than with a BMS ($n=6$). (D) The % area stenosis measured by histomorphometry was significantly smaller in TES compared with BMS ($n=6$).

bovine serum (FBS; SAFC Biosciences, Wicklow, Ireland), and 100 U/mL of penicillin and gentamicin. VSMCs used for experiments were from the fifth to the ninth passages. After 24 h, we exchanged the DMEM containing 1% FBS with or without the addition of 12.5 μM of tacrolimus. The cells without tacrolimus served as a control group. At 48 h of incubation, the cells were harvested by tripsinization and counted in a CDA-500 Particle Analyzer (Sysmex Corporation, Hyogo, Japan).

For Western blotting, rat VSMCs were cultured in 25 cm^2 plates (Agc Techno Glass Co., Ltd., Chiba, Japan) with DMEM containing 1% FBS. After 24 h, we exchanged the DMEM containing 1% FBS with or without the addition of 12.5 μM of tacrolimus. The cells without tacrolimus served as the control group. At 48 h of incubation, cytoplasmic and nuclear proteins were extracted from cultured VSMCs using a Protein and RNA Isolation System. We examined the expression of calcineurin, NFATc4, and IL-2 as described in Section 2.6.

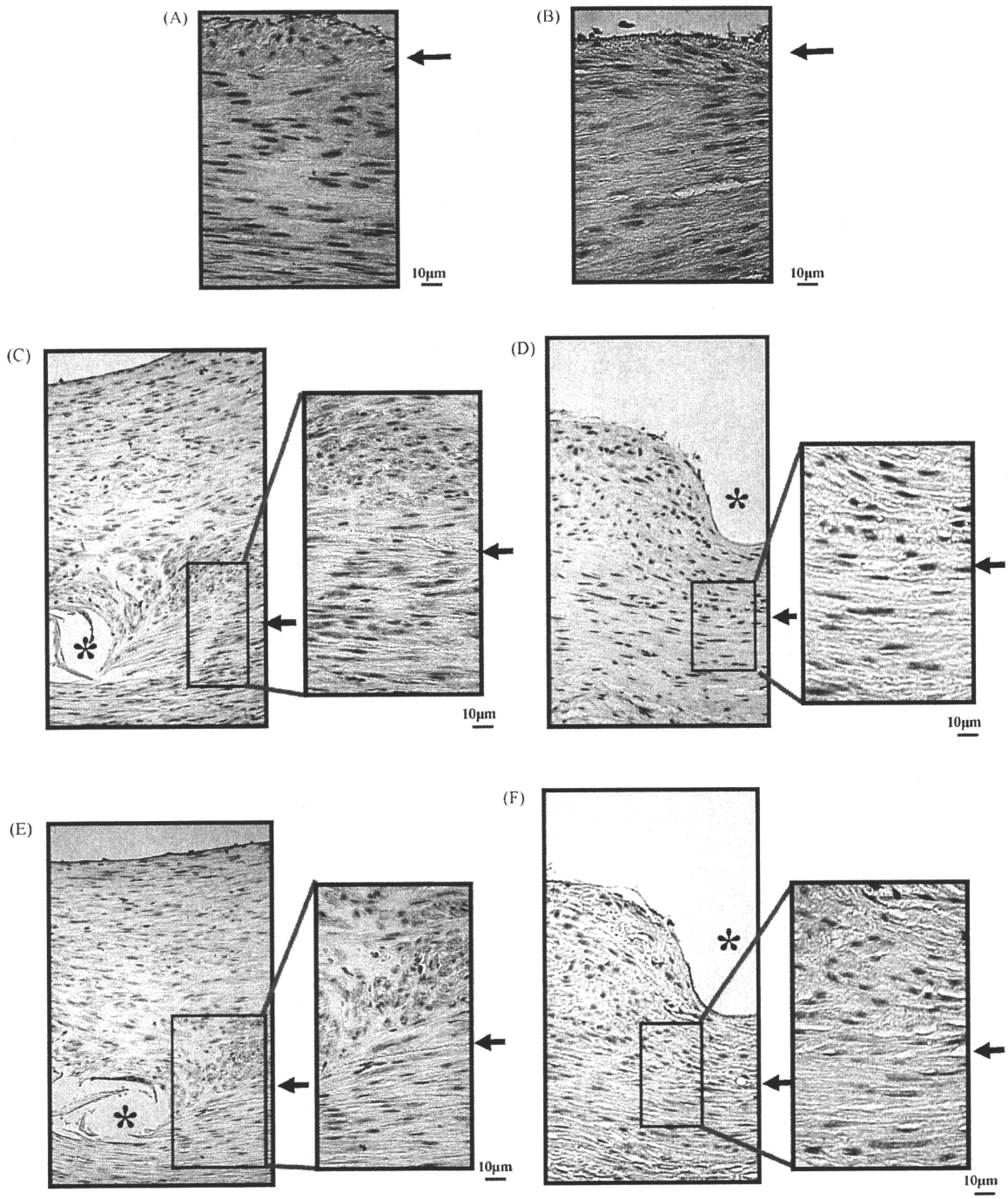

Fig. 3. Immunohistochemical staining for calcineurin in porcine coronary arteries after stenting. Calcineurin was up-regulated in the medial and neointimal smooth muscle cells (SMCs) at 2 (A), 4 (C), and 12 (E) weeks after stenting with a bare metal stent (BMS). In contrast, after stenting with a tacrolimus-eluting stent (TES), the expression of calcineurin was suppressed in the medial and neointimal SMCs at 2 (B), 4 (D) and 12 (F) weeks after stenting. Arrows demonstrate the internal elastic lamina, (*) the space of the stent. Bar, 10 μm.

2.8. Small-interfering RNA transfection

We used small-interfering RNA (siRNA) to suppress the expression of calcineurin. Calcineurin-siRNA (Cat #4390815) and negative control-siRNA (control-siRNA), a 21-nucleotide RNA duplex with no known sequence homology (Cat #4635), were purchased from Ambion (Austin, TX). For siRNA transfection, rat VSMCs (density, 4×10^3/well) were cultured on 48 well plates with medium containing 5% FBS. Transfection of siRNA into cells was achieved by the use of siPORT NeoFX transfection agent and Opti-MEM (Invitrogen) according to the manuals. We mixed the diluted siPORT NeoFX transfection agent and the diluted calcineurin-siRNA, dispensed into cultured wells, and added DMEM containing 5% FBS. 72 h after transfection, we analyzed cell number and isolated protein for Western blotting as described in Section 2.7.

2.9. Statistical analysis

All calculated data are presented as the mean ± SD. Statistical significance was evaluated using unpaired Student's *t*-test for comparisons between two groups. A probability value of $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Quantitative coronary angiography

The coronary arteries of juvenile swine were subjected to implantation of either a BMS or a TES (Fig. 1A). At 12 weeks after stenting, QCA demonstrated that % diameter stenosis of the TES was significantly smaller than that of the BMS (TES: 48.4 ± 13.6% versus BMS: 82.5 ± 16.2%, $P < 0.005$, $n = 6$ in each group) (Fig. 1B).

3.2. Morphometric measurement

Fig. 2A and B shows representative photographs of cross-sections of coronary arteries implanted with a BMS and TES and stained with HE. The histomorphometric measurement showed that the neointimal area was significantly smaller with the TES than with the BMS (TES: 3.87 ± 1.18 mm^2 versus BMS: 5.81 ± 0.70 mm^2, $P < 0.05$, $n = 6$ in each group, Fig. 2C). The % area stenosis measured by histomorphometry was significantly smaller in the TES than the BMS (TES: 45.3 ± 7.2% versus BMS: 64.4 ± 14.7%, $P < 0.05$, $n = 6$ in each group, Fig. 2D).

3.3. Immunohistochemical analysis

Fig. 3 shows the immunohistochemical staining of calcineurin in each group at 2, 4, and 12 weeks after stenting. Immunohistochemical staining demonstrated that calcineurin was not expressed in the medial VSMCs of non-injured coronary arteries (data not shown); it was up-regulated in the medial and neointimal VSMCs at 2, 4, and 12 weeks after stenting with a BMS. In contrast, in coronary arteries implanted with a TES, the protein expression of calcineurin was suppressed in the medial and neointimal VSMCs at 2, 4 and 12 weeks after stenting.

We compared the effect of TES and BMS on re-endothelialization and endothelial function 4 weeks after stenting. To investigate the degree of re-endothelialization, the percentage of vWF-positive length of luminal side was measured [21]. There was no significant difference in the percentage of vWF-positive length between TES and BMS (**Additional Fig. 3A**). In addition, we evaluated endothelial cell function using immunohistochemistry for eNOS. The expression of eNOS in the endothelial cells was similar with TES and BMS (**Additional Fig. 3B**).

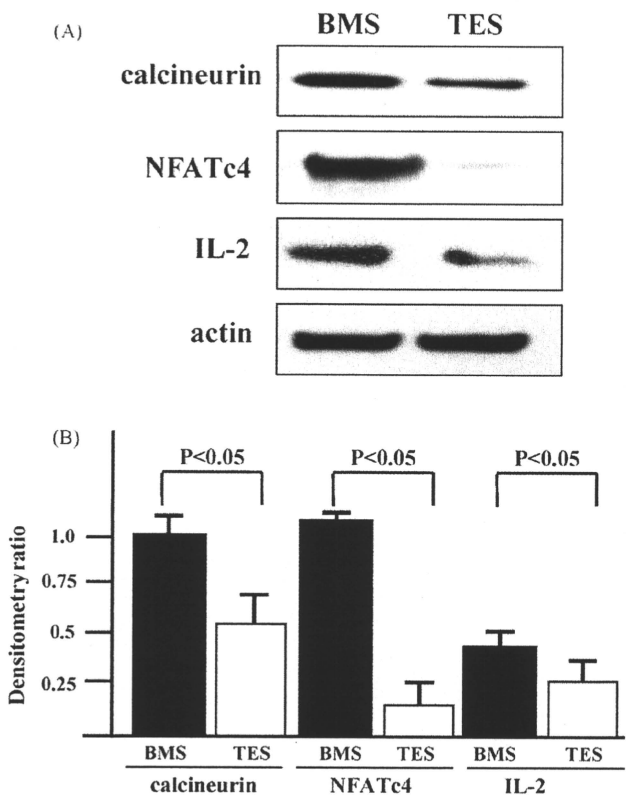

Fig. 4. Western blot analysis for calcineurin, nuclear factor of activated T cell (NFAT), interleukin-2 (IL-2), and actin in neointima of porcine coronary arteries at 4 weeks after implantation of a tacrolimus-eluting stent (TES) or a bare metal stent (BMS). (A) Representative Western blot analysis for calcineurin, NFATc4, IL-2, and actin in BMS and TES. (B) Densitometric analysis of Western blotting demonstrated that the expression of calcineurin, NFATc4, and IL-2 with the TES was significantly lower compared with the BMS.

3.4. Western blot analysis

We isolated protein from porcine coronary arteries at 4 weeks after stenting and used it for Western blotting to analyze the expression of calcineurin, NFATc4, and IL-2. Densitometric analysis of Western blotting demonstrated that the expression of calcineurin, NFATc4, and IL-2 with the TES was significantly lower compared with the BMS (Fig. 4). These results suggested that tacrolimus inhibited the expression of the calcineurin/NFAT/IL-2 pathway that was up-regulated by stenting.

3.5. Cell experiments

In order to confirm the effect of tacrolimus on anti-proliferation of VSMCs and the expression of calcineurin, NFATc4, and IL-2, we performed cell culture experiments. The cell number of the tacrolimus group was significantly lower than that of the control group at 48 h of incubation (tacrolimus group: 12533 ± 176 cells versus control group: 15833 ± 384 cells, $P < 0.05$, Fig. 5A). Densitometric analysis of Western blotting demonstrated that tacrolimus significantly decreased the expression of calcineurin, NFATc4, and IL-2 of cultured VSMC (Fig. 5B and C). We confirmed that tacrolimus inhibited VSMC proliferation and the calcineurin/NFAT pathway *in vitro*.

We confirmed that calcineurin-siRNA but not control-siRNA decreased the protein expression of calcineurin, NFATc4, and IL-2 of cultured VSMC, and demonstrated that the cell number of the VSMCs transfected with calcineurin-siRNA was significantly lower

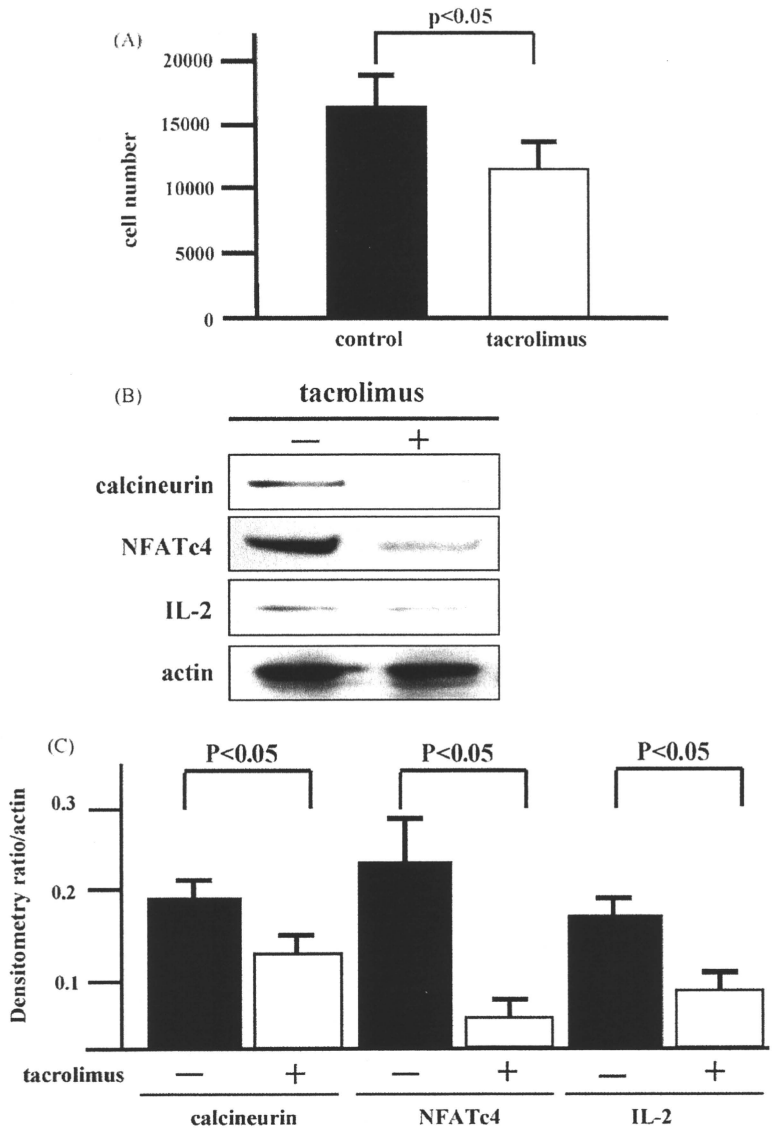

Fig. 5. Cell proliferation at 48 h of incubation with or without tacrolimus and Western blotting for calcineurin, nuclear factor of activated T cell (NFAT), and interleukin-2 (IL-2) of cultured vascular smooth muscle cells (SMCs). (A) The cell number of the tacrolimus group was significantly lower than that of the control group. (B) Representative Western blot analysis for calcineurin, NFATc4, IL-2, and actin in the tacrolimus or control group. (C) Densitometric analysis of Western blotting demonstrated that the expression of calcineurin, NFATc4, and IL-2 in the tacrolimus group was significantly lower compared with the control group.

than that of the VSMCs transfected with control-siRNA (calcineurin-siRNA: 4731 ± 106 cells versus control-siRNA: 7411 ± 233 cells, $P < 0.005$, Additional Fig. 2).

4. Discussion

In this porcine coronary model, the histomorphometric analysis demonstrated that the newly developed TES reduced the neointimal area by 29% compared with a BMS at 12 weeks after stenting. Immunohistochemical staining showed that the expression of calcineurin was up-regulated in neointima and media after stenting, and TES inhibited this up-regulation. In addition, Western blotting demonstrated that the expression of calcineurin, NFATc4, and IL-2 with the TES was lower than with the BMS. Furthermore, in cell culture experiments, we confirmed that tacrolimus and calcineurin-siRNA decreased cell growth and the expression of calcineurin, NFATc4, and IL-2 of cultured VSMCs. It was reported that cyclosporine A suppressed balloon injury-induced neointima formation in a rat carotid artery model by blocking NFAT activation [22]. To the best of our knowledge, this is the first report demonstrating that TES inhibited neointimal formation after stenting via the calcineurin/NFAT signaling pathway.

Intimal thickening and constrictive remodeling are the main elements responsible for restenosis after PCI, and excessive proliferation of VSMCs plays a key role in this process [23]. A previous study reported that tacrolimus inhibited VSMC proliferation [16]. Our cell culture experiments also demonstrated the anti-proliferative effect of tacrolimus on VSMCs. Therefore, we believe that the anti-proliferative effect of tacrolimus on VSMCs results in a reduction of neointimal hyperplasia after stenting.

In activated T cells, tacrolimus and FKBP forms a pentameric complex with calmodulin and calcineurin. Then, this complex inhibits NFAT that is required for activation of the IL-2 gene [14]. Our results of Western blotting demonstrated for the first time that

TES decreased the expression of calcineurin, NFATc4, and IL-2 in the neointima after stenting. Cell culture experiments also revealed that tacrolimus and calcineurin-siRNA decreased the expression of these molecules in cultured VSMCs. Although both tacrolimus and sirolimus bind to FKBP12, the tacrolimus–FKBP complex has been shown to target calcineurin, whereas the sirolimus–FKBP complex targets mammalian target of rapamycin (mTOR) [24]. Therefore, calcineurin may be an important molecular target to prevent restenosis after stenting.

Delayed endothelialization after implantation of a SES or PES may lead to myocardial infarction and death as a result of late stent thrombosis [25,26]. Tacrolimus was reported to have less antiproliferative effect on cultured endothelial cells compared with sirolimus [16]. Therefore, a TES may demonstrate less inhibition of re-endothelialization after implantation than a SES or PES. In our porcine coronary model, the degree of re-endothelialization was similar with TES and BMS. Therefore, tacrolimus is suggested to be a promising compound for the next generation of DESs.

Clinical studies of DESs sometimes show results that are different from the experimental results of long-term porcine studies. The main difference can be explained on the basis of preclinical studies performed in juvenile animals without underlying atherosclerosis [27]. Therefore, a prospective clinical study should be performed in order to evaluate the effect of the newly developed TES on human coronary stenosis.

As the drug is uniformly distributed in the polymer layer in the TES, tacrolimus will be released continuously for several months and completely disappear concomitantly with poly DL-lactate-co-glycolide degradation. The coating method of the TES is completely different from a TES that was developed previously (Janus™, Sorin Biomedica Cardio s.r.l., Saluggia, Italy). The Janus™ stent does not have any polymer vehicle, but has deep reservoirs containing tacrolimus on the external abluminal stent surface; and thus, controlled drug release is difficult. Consequently, the vessel wall may acutely be exposed to an extremely high concentration of the drug, leading to excessive inflammation in the vascular wall. An animal study showed that the tacrolimus concentration in the arterial wall peaked a few days after Janus™ stent implantation and then steeply fell to steady values. These release kinetics may partially explain why the Janus™ stent had a neutral effect on restenosis as shown in the Jupiter II trial [28] and a prospective two-center registry in high-risk patients [29]. Although the same drug was used, the TES may be more useful than the Janus™ stent, because the stent design results in controlled drug release [13].

In conclusion, tacrolimus decreased the proliferation of VSMCs and reduced the expression of calcineurin, NFATc4, and IL-2 in VSMCs. In addition, the newly developed TES inhibited neointimal hyperplasia after stenting via the calcineurin/NFAT/IL-2 signaling pathway, which is one of several mechanisms through which TES inhibits restenosis. Calcineurin may be an important molecular target to prevent restenosis after stent implantation.

Acknowledgments

We wish to thank Mr. Takuji Nishide and Mr. Kohei Fukaya (Kaneka Corporation) for the generous supply of TES and BMS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2009.07.040.

References

[1] Bennett MR. In-stent stenosis: pathology and implications for the development of drug eluting stents. Heart 2003;89(2):218–24.
[2] Ezhov MV, Sumarokov AB, Raimbekova IR, Masenko VP, Naumov VG. Interleukin 6 but not interleukin 10 is associated with restenosis after coronary stenting. Atherosclerosis 2003;169(1):193–4.
[3] Liistro F, Stankovic G, Di Mario C, et al. First clinical experience with a paclitaxel derivate-eluting polymer stent system implantation for in-stent restenosis: immediate and long-term clinical and angiographic outcome. Circulation 2002;105(16):1883–6.
[4] Stefanadis C, Toutouzas K, Stefanadi E. Inhibition of plaque neovascularization and intimal hyperplasia by specific targeting vascular endothelial growth factor with bevacizumab-eluting stent: an experimental study. Atherosclerosis 2007;195(2):269–76.
[5] Deuse T, Erben RG, Ikeno F, et al. Introducing the first polymer-free leflunomide eluting stent. Atherosclerosis 2008;200(1):126–34.
[6] Morice MC, Serruys PW, Sousa JE, et al. Randomized study with the sirolimus-coated Bx velocity balloon-expandable stent in the treatment of patients with de novo native coronary artery lesions. A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. N Engl J Med 2002;346(23):1773–80.
[7] Stone GW, Ellis SG, Cox DA, et al. A polymer-based, paclitaxel-eluting stent in patients with coronary artery disease. N Engl J Med 2004;350(3):221–31.
[8] Moses JW, Leon MB, Popma JJ, et al. SIRIUS Investigators. Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery. N Engl J Med 2003;349(14):1315–23.
[9] Silber S. Paclitaxel-eluting stents: are they all equal? An analysis of six randomized controlled trials in de novo lesions of 3,319 patients. J Interv Cardiol 2003;16(6):485–90.
[10] Stone GW, Moses JW, Ellis SG, et al. Safety and efficacy of sirolimus and paclitaxel-eluting coronary stents. N Engl J Med 2007;356(10):998–1008.
[11] Joner M, Finn AV, Farb A, et al. Pathology of drug-eluting stents in humans: delayed healing and late thrombotic risk. J Am Coll Cardiol 2006;48(1):193–202.
[12] Finn AV, Nakazawa G, Joner M, et al. Vascular responses to drug eluting stents: importance of delayed healing. Arterioscler Thromb Vasc Biol 2007;27(7):1500–10.
[13] Tanimoto S, Giessen WJ, Beusekom HM, et al. MAHOROBA: Tacrolimus eluting coronary stent. Eurointervention 2007;3:149–53.
[14] Thomson AW, Bonham CA, Zeevi A. Mode of action of tacrolimus (FK506): molecular and cellular mechanisms. Ther Drug Monit 1995;17(6):584–91.
[15] Wallemacq PE, Reding R. FK506 (tacrolimus), a novel immunosuppressant in organ transplantation: clinical, biomedical, and analytical aspects. Clin Chem 1993;9(11):2219–28.
[16] Matter CM, Rozenberg I, Jaschko A, et al. Effects of tacrolimus or sirolimus on proliferation of vascular smooth muscle and endothelial cells. J Cardiovasc Pharmacol 2006;48(6):286–92.
[17] Eto H, Biro S, Miyata M, et al. Angiotensin II type 1 receptor participates in extracellular matrix production in the late stage of remodeling after vascular injury. Cardiovasc Res 2003;59(1):200–11.
[18] Schwartz R, Murphy J, Edwards W, Camrud A, Vliestra R, Holmes D. Restenosis after balloon angioplasty: a practical proliferative model in porcine coronary arteries. Circulation 1990;82(6):2190–200.
[19] Eto H, Miyata M, Kume N, et al. Expression of lectin-like oxidized LDL receptor-1 in smooth muscle cells after vascular injury. Biochem Biophys Res Commun 2006;341(2):591–8.
[20] Miyata M, Biro S, Kaieda H, et al. Apolipoprotein J/clusterin is induced in vascular smooth muscle cells after vascular injury. Circulation 2001;104(12):1407–12.
[21] Fukuda D, Enomoto S, Shirakawa I, Nagai R, Sata M. Fluvastatin accelerates re-endothelialization impaired by local sirolimus treatment. Eur J Pharmacol 2009;612(1-3):87–92.
[22] Liu Z, Zhang C, Dronadula N, Li Q, Rao GN. Blockade of nuclear factor of activated T cells activation signaling suppresses balloon injury-induced neointima formation in a rat carotid artery model. J Biol Chem 2005;280(15):14700–8.
[23] Miyata M, Smith JD. Apolipoprotein E allele-specific antioxidant activity and effects on cytotoxicity by oxidative insults and β-amyloid peptide. Nat Genet 1996;14(1):55–61.
[24] Miyata M, Biro S, Kaieda H, Tanaka H. Lipoprotein(a) stimulates the proliferation of cultured human arterial smooth muscle cells through two pathways. FEBS Lett 1995;377(3):493–6.
[25] Virmani R, Guagliumi G, Farb A, et al. Localized hypersensitivity and late coronary thrombosis secondary to a sirolimus-eluting stent: should we be cautious? Circulation 2004;109(6):701–5.
[26] Lang NN, Newby DE. Emerging thrombotic effects of drug eluting stents. Arterioscler Thromb Vasc Biol 2007;27(2):261–2.
[27] Virmani R, Farb A, Kolodgie FD. Histopathologic alterations after endovascular radiation and antiproliferative stents: similarities and differences. Herz 2002;27(1):1–6.
[28] Bartorelli AL, Trabattoni D, Fabbiocchi F, et al. Synergy of passive coating and targeted drug delivery: the tacrolimus-eluting Janus CarboStent. J Interv Cardiol 2003;16(6):499–505.
[29] Romagnoli E, Leone AM, Burzotta F, et al. Outcomes of the tacrolimus drug-eluting Janus stent: a prospective two-centre registry in high-risk patients. J Cardiovasc Med 2008;9(6):589–94.

ELSEVIER

International Journal of Cardiology 141 (2010) 44–48

International Journal of Cardiology

www.elsevier.com/locate/ijcard

Relationship between hyperglycemia and coronary vascular resistance in non-diabetic patients

Hitoshi Ichiki, Shuichi Hamasaki *, Mitsuhiro Nakasaki, Sanemasa Ishida, Akiko Yoshikawa, Tetsuro Kataoka, Masakazu Ogawa, Keishi Saihara, Hideki Okui, Koji Orihara, Takuro Shinsato, Naoya Oketani, Takahiro Shirasawa, Yuichi Ninomiya, So Kuwahata, Shoji Fujita, Takuro Takumi, Yasuhisa Iriki, Satoshi Yoshino, Takehiko Matsushita, Chuwa Tei

Department of Cardiovascular, Respiratory and Metabolic Medicine, Graduate School of Medicine, Kagoshima University, Kagoshima, Japan

Received 11 June 2008; accepted 22 November 2008
Available online 15 January 2009

Abstract

Background: Hyperglycemia upon hospital admission in patients with acute myocardial infarction is associated with the no-reflow phenomenon after successful reperfusion, and increased mortality. However, the mechanism underlying this phenomenon remains unclear. Therefore, the aim of this study was to characterize coronary hemodynamics in a homogenous group of non-diabetic patients without coronary artery disease.

Methods and Results: A total of 104 consecutive non-diabetic patients (mean age, 62±14 years) without coronary artery disease underwent Doppler flow study of the left anterior descending coronary artery. Vascular reactivity was examined by intra-coronary administration of papaverine, acetylcholine (Ach), and nitroglycerin using a Doppler guidewire. Coronary vascular resistance (CVR) was calculated as the mean arterial pressure divided by coronary blood flow (CBF). Baseline CVR was shown as CVR at control and minimal CVR was shown as CVR with papaverine administration. Fasting plasma glucose (FPG) level had a significant, positive correlation with baseline CVR and minimal CVR ($r=0.24$, $p<0.02$ and $r=0.21$, $p<0.05$, respectively). Hemoglobin A1c (HbA1c) also had a significant, positive correlation with baseline CVR and minimal CVR ($r=0.31$, $p<0.01$ and $r=0.32$, $p<0.01$, respectively). The percent change in CBF induced by Ach was inversely correlated with HbA1c but not with FPG ($r=0.22$, $p<0.05$ and $r=0.06$, $p=0.57$, respectively). By contrast, neither FPG nor HbA1c had significant correlation with coronary flow reserve to papaverine.

Conclusion: These data demonstrate that elevated glucose levels are associated with increases in baseline and minimal coronary vascular resistance. These changes may contribute to unfavorable coronary hemodynamics in non-diabetic patients without coronary heart disease.

Keywords: Coronary vascular resistance; FBS; HbA1; Hyperglycemia

1. Introduction

Hyperglycemia upon hospital admission in patients with acute myocardial infarction (AMI) is associated with the no-reflow phenomenon after successful reperfusion [1], resulting in larger infarct size and worse functional recovery. Further, hyperglycemia in patients with ST-segment elevation acute myocardial infarction is an important predictor of impaired epicardial flow before reperfusion therapy [2], and hyperglycemia in patients with AMI is associated with increased mortality [3–7]. However, the mechanisms underlying these adverse effects of hyperglycemia remain unknown. Therefore, the aim of this study was to characterize coronary hemodynamics in a

* Corresponding author. Department of Cardiovascular, Respiratory and Metabolic Medicine, Graduate School of Medicine, Kagoshima University, 8-35-1, Sakuragaoka, Kagoshima 890-8520, Japan. Tel.: +81 99 275 5318; fax: +81 99 265 8447.
E-mail address: hamasksh@m.kufm.kagoshima-u.ac.jp (S. Hamasaki).

0167-5273/$ - see front matter © 2008 Published by Elsevier Ireland Ltd.
doi:10.1016/j.ijcard.2008.11.148

Table 1
Clinical characteristics of the study patients.

Age (years)	62±14 (17–85)
Male gender	69 (66%)
Risk factors	
Hypertension	57 (55%)
Hyperlipidemia	35 (34%)
Smoker	27 (26%)
Laboratory data	
FPG (mg/dl)	94±8 (74–120)
HbAlc (%)	5.2±0.4 (4.1–6.3)
Total-cholesterol (mg/dl)	197±37 (114–314)
Triglycerides (mg/dl)	122±64 (40–368)
HDL-cholesterol (mg/dl)	57±15 (28–98)
LDL-cholesterol (mg/dl)	113±28 (53–211)

HDL: high density lipoprotein; LDL: low density lipoprotein; Values are mean±SD.

homogenous group of non-diabetic patients without coronary artery disease.

2. Methods

2.1. Study population

A total of 187 consecutive non-diabetic patients who had been referred for cardiac catheterization to exclude coronary artery disease were considered for enrollment in this study. Of these, 104 patients met the following inclusion criteria: 1) angiographically smooth arteries; 2) mild irregularities, <30% lumen diameter stenosis by visual assessment in any major conduit vessel; 3) proximal coronary arteries >2.0 mm in diameter; and 4) lacking a history of previous myocardial infarction, previous coronary revascularization, valvular heart disease, variant angina, cardiomyopathy, or myocarditis.

Patients meeting the following criteria were considered to have obvious diabetes and excluded: 1) previous diagnosis of diabetes, 2) current treatment by oral hypoglycemic agents or insulin, or 3) concentration of fasting plasma glucose (FPG) >126 mg/dl or hemoglobin A1c (HbA1c) >6.5% at admission [8].

Informed consent was obtained from each patient and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the institution's human research committee.

2.2. Study protocol

Diagnostic coronary angiography was performed using a 6F Judkins catheter with a standard femoral percutaneous approach. Heparin (5000 units) was administered at the beginning of the procedure. Non-ionic contrast material was used for all patients. No nitroglycerin was given prior to the diagnostic procedure. Coronary blood flow (CBF) response to papaverine, acetylcholine (Ach), and nitroglycerin was studied according to previous reports [9,10]. After control coronary angiograms, interventions were performed as follows: 1) a 0.014-inch Doppler guidewire (Cardiometrics, Santa Anna, CA, USA) was introduced into the left anterior descending coronary artery; 2) after obtaining a stable Doppler signal, a bolus of papaverine (an endothelium-independent vasodilator in resistance coronary arteries) (12.5 mg/5 ml) was injected through a catheter; 3) infusion of Ach (an endothelium-dependent vasodilator in resistance and conduit coronary arteries) (0.5 ml/min) at a dose of 3 μg/min for 2 min was performed via the catheter [11,12,4] a bolus of nitroglycerin (an endothelium-independent vasodilator in conduit coronary arteries) (200 μg/5 ml) was administered. Drugs were infused with a minimum 5-min interval. Coronary arteriography was performed before and 2 min after each dose of Ach and after administration of nitroglycerin. Phasic CBF velocities, arterial blood pressure, and heart rate were monitored continuously and recorded. Measurements obtained during steady state conditions were used as control values for later analysis.

Doppler flow velocity spectra were analyzed on-line to determine time-averaged peak velocity. Volumetric CBF was determined from the formula: CBF=cross-sectional area×-average peak velocity×0.5 [13]. Coronary flow reserve to papaverine was calculated as the ratio of maximal CBF induced by papaverine to basal CBF, which was equivalent to

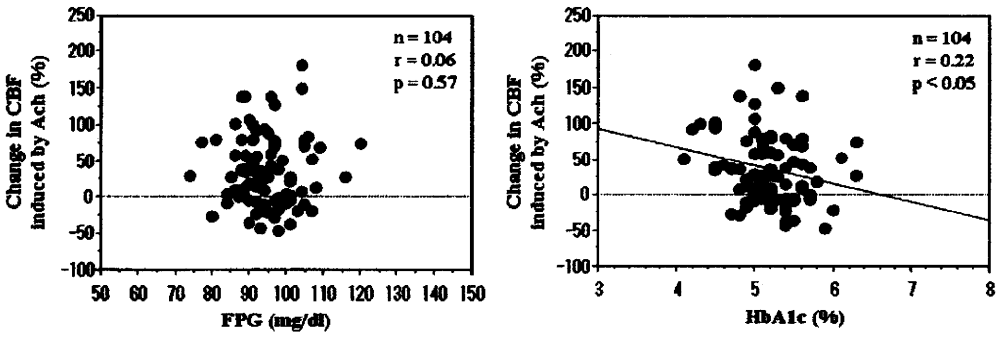

Fig. 1. Scattergram illustrating the correlation between percent change in CBF induced by Ach and FPG (left panel) and HbA1c (right panel).

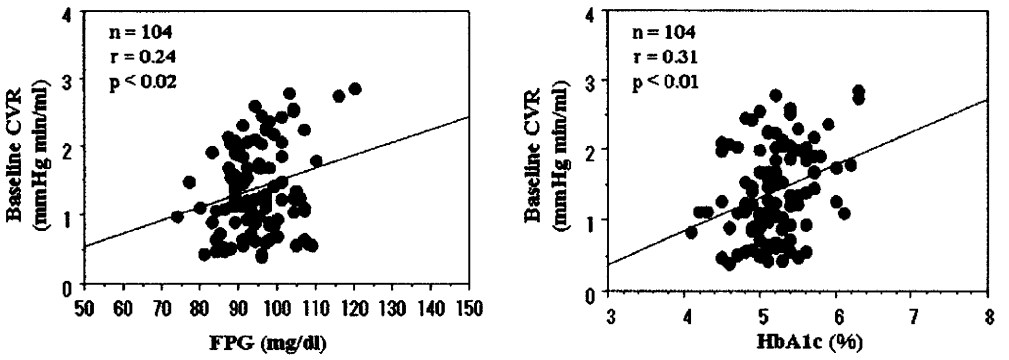

Fig. 2. Scattergram illustrating the correlation between baseline CVR and FPG (left panel) and HbA1c (right panel).

the endothelium-independent function of the resistance coronary artery. Endothelium-dependent function was calculated as the percent increase of CBF in response to Ach [9,14].

Coronary vascular resistance (CVR) was calculated by mean arterial pressure divided by CBF. Baseline CVR is shown as CVR at control, and minimal CVR is shown as CVR with papaverine administration.

2.3. Statistics

All data are expressed as the mean value±SD. One-way analysis of variance (ANOVA) was used for comparison of continuous variables, and significance of difference was calculated using the Scheffe F test. Differences were considered significant at $p<0.05$. Statistical analysis was performed with Statview, ver. 5.0 (SAS Institute Inc., Cary, NC).

3. Results

A total of 104 patients were evaluated. Patient characteristics are shown in Table 1. All patients enrolled in this study had concentrations of FPG<126 mg/dl and HbA1c<6.5%.

3.1. Changes in coronary blood flow

The relationship between FPG, HbA1c, and percent change in CBF induced by Ach are shown in Fig. 1. Percent change in CBF induced by Ach (i.e., namely endothelium-dependent vasodilatation in resistance arteries) was inversely correlated with HbA1c but not with FPG, which suggests that chronically higher levels of glucose concentration are associated with impaired endothelial function in resistance coronary arteries in non-diabetic patients. Coronary flow reserve to papaverine (i.e., endothelium-independent vasodilatation in resistance arteries) did not correlate with FPG or HbA1c ($r=0.08$, $p=0.42$; $r=0.02$, $p=0.85$, respectively) in non-diabetic patients.

3.2. Changes in coronary artery diameter

Neither FPG nor HbA1c was correlated with percent change in coronary artery diameter (CAD) induced by Ach (i.e., namely endothelium-dependent vasodilatation in epicardial arteries) ($r=0.01$, $p=0.97$ and $r=0.03$, $p=0.82$, respectively). Similarly, percent change in CAD induced by nitroglycerin (i.e., namely endothelium-independent vasodilatation in epicardial arteries) did not correlate with FPG or HbA1c ($r=0.01$, $p=0.94$ and $r=0.06$, $p=0.57$, respectively). Thus, neither FPG nor HbA1c were correlated with endothelium-dependent and -independent vasodilation of the epicardial coronary arteries in non-diabetic patients.

3.3. Coronary vascular resistance

The relationship between baseline CVR and minimal CVR to FPG and HbA1c are shown in Figs. 2 and 3, respectively.

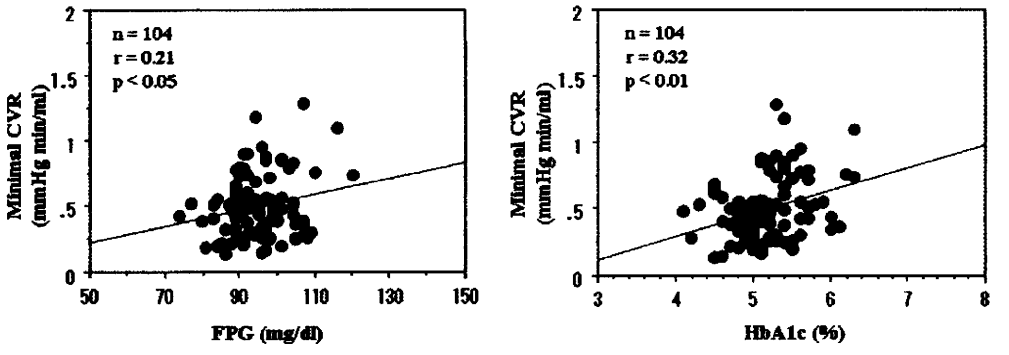

Fig. 3. Scattergram illustrating the correlation between minimal CVR and FPG (left panel) and HbA1c (right panel).

FPG and HbA1 had a significant, positive correlation with baseline CVR and minimal CVR. These results suggest that acute and chronic high glucose concentrations are associated with increased vascular resistance in coronary arteries at baseline and at a hyperemic state in non-diabetic patients.

4. Discussion

The present study demonstrated that chronic exposure to high glucose concentrations is associated with coronary endothelial dysfunction and that baseline and minimal CVR are elevated in the context of acute and chronic hyperglycemia in non-diabetic patients without coronary artery disease. These data suggest that elevated blood glucose concentrations may contribute to impaired coronary flow in non-diabetic patients.

4.1. Hyperglycemia and coronary endothelial function

Previous studies have reported that chronic hyperglycemia results in impairments in endothelium-dependent coronary artery vasodilation [15,16], which is consistent with results from the present study. Further, other studies have demonstrated that acute hyperglycemia results in impaired coronary flow via attenuation of mitochondrial ATP—sensitive potassium channel (K_{ATP} channel) activation and via promotion of platelet-dependent thrombus formation [17,18]. By contrast, other investigators have reported that hyperglycemia-induced vascular damage is mediated by increased production of oxygen free radicals, including superoxide anion, from endothelial cells [19–21]. Increased superoxide anion can inactivate nitric oxide and result in enhanced contractility and proliferation of vascular smooth muscle cells with increased vasomotor tone, platelet hyper-reactivity [22], alteration of the adhesive properties of the endothelium [23], and increased production of cytokines [24].

4.2. Hyperglycemia and coronary vascular resistance

Farouque et al. [25] suggested that K_{ATP} channels contribute to basal CVR by demonstrating that inhibition of these channels resulted in impairments in resting CVR. Further, acute hyperglycemia prevents the positive effect of ischemic preconditioning, probably through the attenuation of mitochondrial K_{ATP} channel activity. Barbagallo et al. [26] reported that glucose increased intracellular calcium content in vascular smooth muscle, while other investigators reported that FPG and HbA1c levels are both closely related to fasting basal cytosolic free calcium levels [27]. These observations are consistent with results from the present study and may provide a mechanistic link between why acute hyperglycemia and impaired coronary flow.

In this study, patients with obvious diabetes were excluded. Kawano et al. [28] demonstrated glucose-induced impairments in endothelium-dependent vasodilation in patients with IGT, even when FRG levels were within a normal limit. Further, this effect persisted at 2 h after glucose loading. Thus, repeated exposure to postprandial hyperglycemia may play an important role in the vascular dysfunction, even in our patients without obvious diabetes.

4.3. Limitations

This study possesses several limitations. First, the study population included only patients with normal or mildly diseased coronary arteries. Thus, the present findings may not be applicable to patients with advanced coronary artery disease. Second, the present study was based on a retrospective analysis of the patients and was only a descriptive study in which we established an association between elevated blood glucose concentrations and an increase of CVR; a more prospective study is required to determine the effect of glycemic control on CVR.

5. Conclusion

Increases in baseline and minimal CVR in association with acute or chronic hyperglycemia may contribute to unfavorable coronary hemodynamics and mortality in non-diabetic patients.

Acknowledgment

The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [29].

References

[1] Iwakura K, Ito H, Ikushima M. Association between hyperglycemia and the no-reflow phenomenon in patients with acute myocardial infarction. J Am Coll Cardiol 2003;41:1–7.
[2] Timmer JR, Ottervanger JP, de Boer MJ, et al. Hyperglycemia is an important predictor of impaired coronary flow before reperfusion therapy in ST-segment elevation myocardial infarction. J Am Coll Cardiol 2005;45:999–1002.
[3] Capes SE, Hunt D, Malmberg K, Gerstein HC. Stress hyperglycaemia and increased risk of death after myocardial infarction in patients with and without diabetes: a systematic overview. Lancet 2000;355:773–8.
[4] Suleiman M, Hammerman H, Boulos M, et al. Fasting glucose is an important independent risk factor for 30-day mortality in patients with acute myocardial infarction: a prospective study. Circulation 2005;111:754–60.
[5] Kosuge M, Kimura K, Ishikawa T, et al. Persistent hyperglycemia is associated with left ventricular dysfunction in patients with acute myocardial infarction. Circ J 2005;69:23–8.
[6] Kosuge M, Kimura K, Kojima S, et al. Effects of glucose abnormalities on in-hospital outcome after coronary intervention for acute myocardial infarction. Circ J 2005;69:375–9.
[7] Timmer JR, van der Horst IC, de Luca G, et al. Zwolle Myocardial Infarction Study Group. Comparison of myocardial perfusion after successful primary percutaneous coronary intervention in patients with ST-elevation myocardial infarction with versus without diabetes mellitus. Am J Cardiol 2005;95:1375–7.
[8] Kuzuya T, Nakagawa S, Satoh J, et al. Committee of the Japan Diabetes Society on the diagnostic criteria of diabetes mellitus. Report of the committee on the classification and diagnostic criteria of diabetes mellitus. Diabetes Res Clin Pract 2002;55:65–85.