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Effects of Pitavastatin on Fasting and Postprandial Endothelial Function and Blood Rheology in Patients With Stable Coronary Artery Disease

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Background: Because postprandial hypertriglyceridemia and hyperglycemia may promote atherosclerosis, the present study investigated the effects of a clinical dose of pitavastatin on endothelial function and blood rheology in patients with coronary artery disease (CAD) before and after eating a test meal.

Methods and Results: The 16 patients with stable CAD and mild dyslipidemia and 6 age-matched healthy men as controls were recruited. In each group, forearm blood flow (FBF) was measured during postischemic reactive hyperemia and blood samples were taken before and 2 h after the test meal. Urinary 8-hydroxy-2`-deoxyguanosine (8-OHdG) was also measured. The patients were started on pitavastatin 2 mg/day. The tests were repeated after 6 months. Maximum FBF during hyperemia in the baseline fasting phase was significantly lower in CAD patients than in control subjects (P=0.040). Fasting and postprandial FBF during reactive hyperemia significantly improved after pitavastatin treatment (P<0.05 vs baseline data for each phase) associated with reduced urine 8-OHdG, increased plasma adiponectin and improved lipid profile. No significant differences in baseline rheological parameters were seen between controls and CAD patients.

Conclusions: Pitavastatin significantly improved fasting and postprandial dyslipidemia and endothelial dysfunction in CAD patients, partly via reducing oxidative stress and increasing plasma adiponectin, although rheological parameters remained unchanged. (*Circ J* 2009; **73:** 1523–1530)

Key Words: Angina pectoris; Endothelial function; Oxidative stress; Postprandial dyslipidemia; Rheology

Accumulating evidence shows that postprandial metabolic changes play an important role in the development of atherosclerotic cardiovascular disease[1] Two recent cohort studies have reported that postprandial hypertriglyceridemia is associated with increased risk of coronary vascular events[1,2] In addition, postprandial hyperglycemia in patients with diabetes mellitus (DM) or impaired glucose tolerance has been identified as an independent risk factor for cardiovascular disease[3,4] and a stronger predictor of cardiovascular mortality than fasting plasma glucose[5] High-calorie cooked meals, rich in processed carbohydrates and saturated fat, can lead to both hyperglycemia and hypertriglyceridemia with elevated serum levels of triglyceride (TG)-rich remnant lipoproteins[6] The resultant endothelial and hemorheological dysfunction may be closely related to transiently enhanced postprandial oxidative stress, one of the relevant therapeutic targets for statins[7–9] Statins reduce cardiovascular events in dyslipidemic patients for both primary[10,11] and secondary prevention[12] of coronary arterial events by lowering of lipid levels, including the oxidized low-density lipoprotein-cholesterol (LDL-C) involved in atherosclerosis, and via pleiotropic effects, because mevalonate is a precursor of various nonsteroidal isoprenoid products as well as cholesterol. Effects of strong statins on postprandial endothelial function, blood rheology and adiponectin in patients with dyslipidemia or DM have been reported[13–16] but effects in patients with coronary artery disease (CAD) are not fully understood[17] Such information might be valuable in constructing better therapeutic strategies for patients with CAD.

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The present study investigated effects of a clinical dose of pitavastatin, a strong hydrophobic statin providing marked improvements in lipids (including increases in high-density lipoprotein-cholesterol (HDL-C)), postprandial lipids, endothelial function, blood rheology, and oxidative stress in Japanese patients with CAD.

Methods

Study Subjects

Entry criteria for CAD patients were age between 40 and 75 years and evidence of significant coronary artery stenosis (>70%) by coronary arteriography within the previous 5 years at Saitama Medical Center of Jichi Medical University. Exclusion criteria included unstable angina; previous

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Table 1. Clinical Characteristics of Subjects

	Control (n=6)	CAD (n=16)	P value
Age (years)	57.0±8.3	63.2±7.8	0.137
Hypertension (%)	0 (0%)	10 (62.5%)	0.009
Hyperlipidemia (%)	0 (0%)	9 (56.3%)	0.017
Diabetes mellitus (%)	0 (0%)	9 (56.3%)	0.017
Ex-smoker (%)	4 (66.7%)	10 (62.5%)	0.856
BMI	22.1±2.6	23.5±3.2	0.619
Fasting blood test			
TC (mg/dl)	176.8±18.8	218.2±34.3	0.012
LDL-C (mg/dl)	97.7±21.2	139.9±26.3	0.002
HDL-C (mg/dl)	55.0±6.5	40.6±7.5	<0.001
LDL-C/HDL-C	1.82±0.53	3.53±0.81	<0.001
Triglycerides (mg/dl)	81.7±38.9	160.6±45.9	0.001
RLP-C (mg/dl)	3.13±0.76	5.78±2.30	0.013
FFA (mmol/L)	0.46±0.11	0.47±0.13	0.880
Adiponectin (μg/ml)	10.55±1.65	6.19±2.76	<0.001
hsCRP (mg/L)	1.00±0.80	1.80±1.70	0.286
HbA1c (%)	5.03±0.10	6.30±1.24	0.028
Plasma glucose (mg/dl)	102.2±8.2	120.4±26.7	0.121
IRI (μU/ml)	6.6±2.6	8.6±3.9	0.253
Hematocrit	41.5±2.3	44.1±4.3	0.180
Blood passage time (s)	44.7±8.0	46.8±7.9	0.595
Basal FBF	5.1±0.45	6.0±1.4	0.141
Max. FBF during RH	36.7±4.6	28.8±8.2	0.040
FBF after NTG s.l. (ml·min^{-1}·100 ml tissue^{-1})	7.7±1.5	6.8±1.8	0.096

Values are mean±SD or the percentage of participants in that category.
CAD, coronary artery disease; BMI, body mass index; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; RLP-C, remnant-like particle cholesterol; hsCRP, high-sensitivity C-reactive protein; Hb, glycohemoglobin; IRI, serum insulin; FBF, forearm blood flow; Max., maximum; RH, reactive hyperemia; NTG, nitroglycerine; s.l., sublingual.

history of coronary artery bypass surgery; left ventricular ejection fraction <50%; renal dysfunction defined as serum creatinine >1.3 mg/dl; current smoker; cerebrovascular event within the previous 3 months; coronary revascularization by catheter angioplasty within the previous 3 months; chronic heart failure; DM uncontrolled by diet with hemoglobin (Hb) A1c ≥7.5%; LDL-C ≥180 mg/dl or <100 mg/dl, and taking lipid-lowering agents, angiotensin-converting enzyme inhibitors, nitrates or blood sugar-lowering agents including insulin or supplemental vitamins. A total of 29 patients were selected as candidates according to these criteria. Of them, 13 patients declined to participate in the study. Finally, we recruited 16 Japanese male patients with CAD and 6 age-matched healthy male volunteers (controls); their characteristics are shown in **Table 1**. All 16 CAD patients were taking aspirin, 8 were taking calcium-channel blockers, 7 were taking angiotensin II receptor blockers, 2 were taking β-blockers, and 4 were taking ticlopidine. Four patients had a history of myocardial infarction and 7 had a history of percutaneous coronary intervention using stents. All control subjects had normal blood pressure, were not taking any medications and showed normal results on routine physical examination and standard laboratory tests, including serum lipid profile and plasma glucose concentrations. Written informed consent was given by all subjects before examination. Medications for CAD patients other than pitavastatin were not altered during the study period. All subjects were advised to maintain their usual lifestyle during the study period of 6 months.

Study Protocol

The protocol was approved by the Ethics Committee of Jichi Medical University. This was a prospective observational study in a single institution comparing pitavastatin intervention in CAD patients with normal control subjects. Because of ethical concerns, no CAD patient without statins was included. Subjects fasted overnight, abstained from beverages containing alcohol or caffeine and did not take any medications for at least 12 h before laboratory measurements. After subjects arrived at the laboratory room at 08.30 h, fasting blood samples were taken for assessment of blood rheology and the following: HbA1c; serum total cholesterol (TC); TG; LDL-C; HDL-C; remnant-like particle cholesterol (RLP-C), plasma glucose, insulin and high-sensitive C-reactive protein (hsCRP); and plasma adiponectin. Urine samples were also obtained for measurement of 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels. Forearm blood flow (FBF) following reactive hyperemia was then measured using strain-gauge plethysmography on the right forearm to assess endothelial function. All subjects then ate within 30 min a cooked test meal (a modified standard test meal as proposed by a working group of the Japan Diabetes Society) comprising buttered and honeyed toast, boiled egg, yogurt and vegetable soup (680 kcal: carbohydrates 60 g; protein 20 g; fat 40 g (unsaturated fatty acids, 13 g))[18] All measurements, except HbA1c and adiponectin, were repeated 2 h after taking the test meal. After measurements at baseline, all patients with CAD started their intake of pitavastatin (Kowa, Tokyo, Japan) of 2 mg daily. The tests were repeated after 6 months in each group with the exception of urinary sampling for control subjects.

Blood and Urine Sampling and Assay

Serum levels of TC, TG, LDL-C, HDL-C and plasma glucose were measured using enzymatic methods. RLP-C was measured with 0.2-ml serum samples using an enzyme-linked immunosorbent assay kit (JIMRO; Otsuka Pharmaceutical, Osaka, Japan). HbA1c was measured by high-

Table 2. Serial Changes in Laboratory Data in Patients With CAD (n=16)

	Baseline		6 months	
	Preprandial	Postprandial	Preprandial	Postprandial
BMI	23.5±3.2		24.1±3.2	
TC (mg/dl)	218.2±34.3	209.4±33.0	164.5±27.5**	155.1±22.9**
HDL-C (mg/dl)	40.6±7.5	38.5±6.8	48.9±7.9**	46.2±7.2**
LDL-C (mg/dl)	139.9±263	132.9±24.4	90.2±18.7**	82.5±14.5**
LDL-C/HDL-C	3.53±0.81	3.54±0.82	1.87±0.38**	1.81±0.32**,##
TG (mg/dl)	160.6±45.9	231.3±59.9**	113.8±48.4*	179.5±61.3#
RLP-C (mg/dl)	5.8±2.3	9.5±3.2**	3.9±1.7*	6.3±2.5#,§
FFA (mmol/L)	0.47±0.13	0.29±0.11*	0.43±0.20	0.31±0.11*,§
Adiponectin (μg/ml)	6.19±2.76		7.45±3.33*	
Plasma glucose (mg/dl)	120.4±26.7	191.9±61.6**	124.6±31.1	186.6±70.0**
IRI (μU/ml)	8.6±3.9	62.8±31.1**	8.8±3.8	47.7±18.5**
hsCRP (mg/L)	1.80±1.70	1.70±1.60	1.50±1.90	1.30±1.70
HbA_{1c} (%)	6.3±1.2		6.5±1.2	
Hematocrit	44.1±4.3	42.8±3.6	42.8±3.4	41.3±3.2
Blood passage time (s)	46.8±7.9	46.9±10.7	44.9±8.1	42.2±9.1
Basal FBF	4.1±1.3	5.1±2.2*	4.6±2.6	6.8±3.2*,§
FBF after NTG s.l. (ml·min^{-1}·100 ml tissue^{-1})		6.8±1.8		7.2±3.4

*P<0.05 vs preprandial baseline data, **P<0.01 vs preprandial baseline data, #P<0.05 vs postprandial baseline data, ##P<0.01 vs postprandial baseline data, §P<0.05 vs preprandial data at 6 months.
Abbreviations as in Table 1.

performance liquid chromatography, serum insulin levels by enzyme immunoassay, and serum hsCRP by a monoclonal antibody-based latex agglutination test that allows measurement of samples with low (0.01 mg/dl) to high (42 mg/dl) concentrations (Nanopia CRP; Daiichi Pure Chemical, Tokyo, Japan).[19] Plasma adiponectin levels were measured using an enzyme-linked immunosorbent assay kit (Otsuka Pharmaceutical, Osaka, Japan).[20] Oxidative damage to DNA and its precursors were measured by determining 8-OHdG levels in urine. Urine samples were stored in polypropylene tubes at −80°C. Urinary 8-OHdG levels were measured using a competitive enzyme-linked immunosorbent assay kit (New 8-OHdG Check; Japan Institute for the Control of Aging, Nikken SEIL, Fukuroi, Japan). Levels of urinary 8-OHdG were expressed as a ratio corrected by urinary creatinine levels.

Blood Kinetics in Narrow Microchannels Ex Vivo

Fasting blood samples were carefully taken from the left cubitus vein into a 5-ml syringe containing heparin sodium (50 units/ml blood). The passage time for 0.1 ml of blood through microchannels (8700-parallel, 7-μm equivalent diameter, 20-μm-long channels; Kowa) under a constant suction pressure of −20 cmH_2O was determined using a Microchannel Flow Analyzer (Kowa) as a whole blood rheological parameter.[18,21,22] Saline passage time was determined before each blood measurement for calibration.

Noninvasive Assessment of Endothelial Function

Endothelial function was assessed by FBF in response to reactive hyperemia using strain-gauge plethysmography as described elsewhere.[18,23,24] In brief, patients remained seated in a quiet, air-conditioned room (constant temperature, 23–24°C) throughout the procedure. FBF measurements were deliberately made contralateral to the arm from which blood had been drawn. A strain-gauge was attached to upper part of the forearm and connected to a plethysmography device (EC-5R; D.E. Hokanson Issaquah, WA, USA), and supported at the height of the right atrium. A wrist cuff was inflated to 50 mmHg above systolic blood pressure 1 min before first measurement of basal flow and throughout the measurement of FBF to exclude hand circulation from the measurement. The upper arm cuff was inflated to 40 mmHg for 7 s in each 15-s cycle to occlude venous outflow from the arm using a rapid cuff inflator (EC-20; D.E. Hokanson). The blood flow output signal was transmitted to a recorder (U-228; Advance, Nagoya, Japan). FBF was expressed as milliliters per minute per 100 ml of forearm tissue volume. Five plethysmographic measurements were averaged to obtain basal FBF values and FBF after nitroglycerine (NTG) administration.

To induce reactive hyperemia, the upper right arm cuff was inflated to 200 mmHg for 5 min. FBF was continuously measured for 3 min after cuff release. Only in the postprandial phase, 30 min after reactive hyperemia, was the FBF measurement conducted with sublingual administration of 0.3 mg of NTG.

Coronary risk factors were identified from the medical history or from the following data: HbA_{1c} level ≥6.5% for DM; TC level ≥220 mg/dl for dyslipidemia; and systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg for hypertension.

Statistical Analysis

Data were statistically analyzed using JMP 7.01 J software (SAS Institute, Cary, NC, USA). Continuous variables are described as mean±standard deviation (SD), and where appropriate, Student's t-test or 2-way analysis of variance followed by the Tukey-Kramer post-hoc test were used. Categorical variables are described as frequencies and were compared using Fisher's exact test. Probability values of $P<0.05$ were considered to indicate statistical significance.

Results

All subjects completed the study protocol without any cardiovascular events. Pitavastatin was continued until the end of the study in all subjects and no adverse effects occurred in the CAD patients. In 1 patient, β-blocker administration was stopped because of sinus bradycardia. In all other patients, medications were unaltered during the study period. At baseline, serum levels of TC, LDL-C, LDL-C/

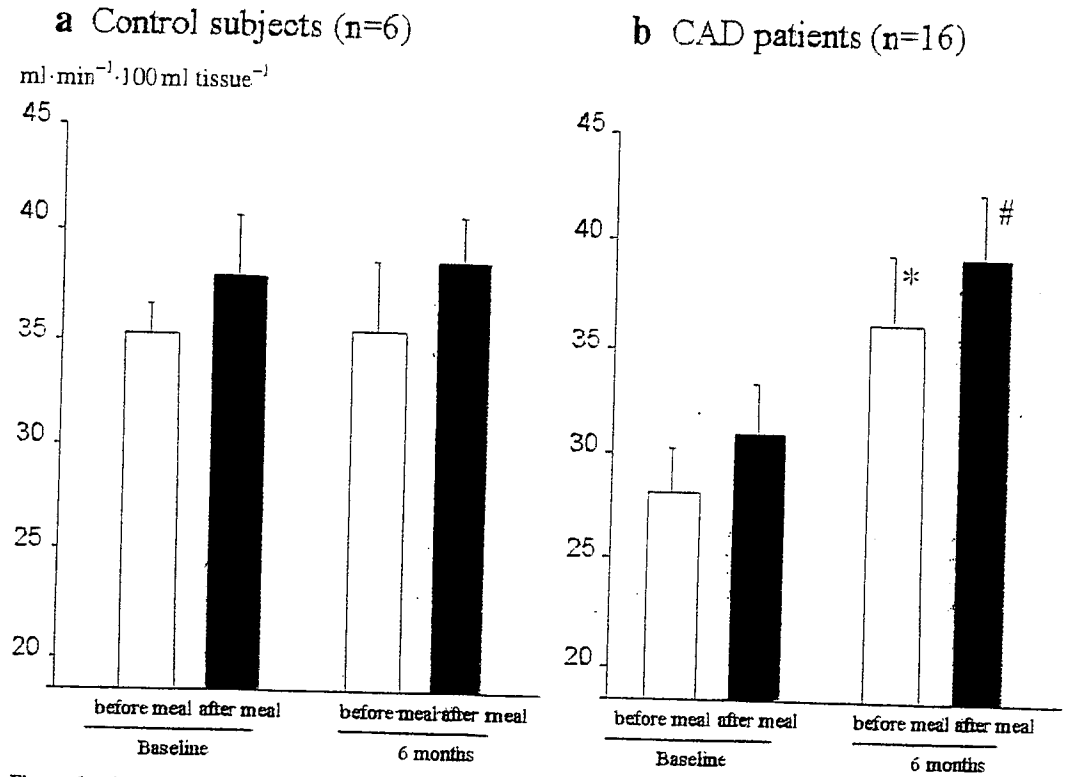

Figure 1. Maximum forearm blood flow (FBF) following postischemic reactive hyperemia in control subjects (a, n=6) and patients with stable angina pectoris (b, n=16) before (white bar) and 2 h after intake of the test meal (black bar). Pre- and postprandial FBF following reactive hyperemia were significantly increased after 6-month pitavastatin treatment in patients with angina pectoris (b). In control subjects, FBF following reactive hyperemia remained unchanged after the period of 6 months. *P<0.05 vs baseline preprandial data; #P<0.05 vs baseline postprandial data. CAD, coronary artery disease.

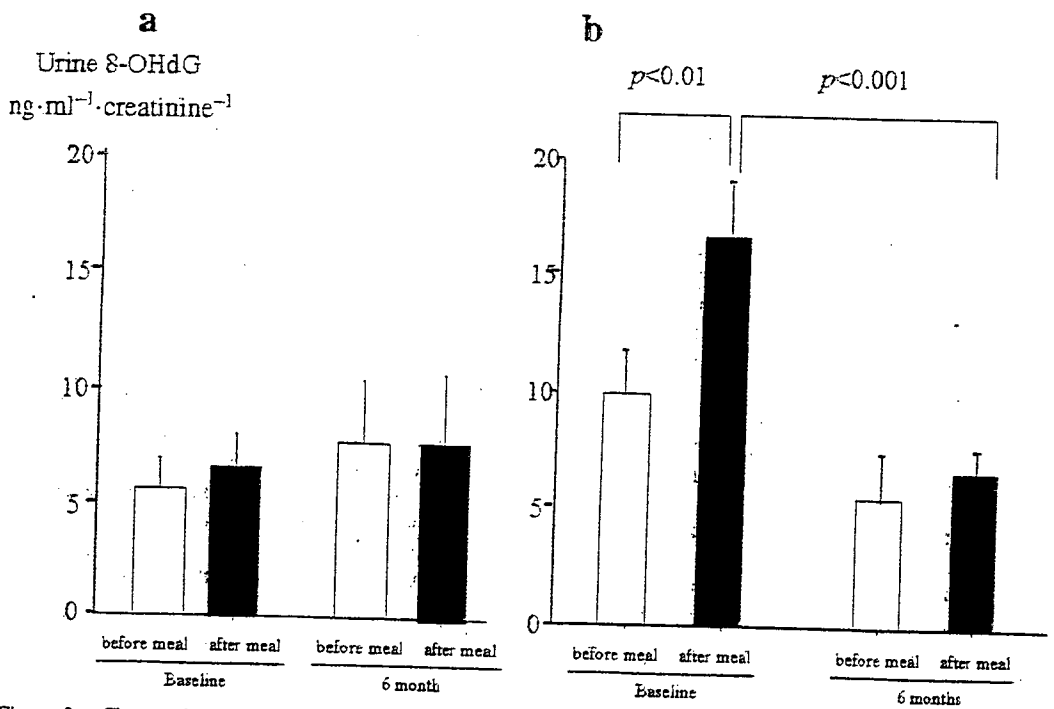

Figure 2. Changes in urine 8-hydroxy-2'-deoxyguanosine (8-OHdG) as a parameter of oxidative stress in control subjects (a, n=6) at baseline and in patients with stable angina pectoris (b, n=16) before (white bar) and 2 h after intake of the test meal (black bar). In patients with angina pectoris, urinary 8-OHdG levels were significantly increased at postprandial phase compared with fasting data (P<0.01), although those levels remained unchanged in the control subjects. Note that postprandial urine 8-OHdG level was significantly decreased compared with baseline postprandial data in patients with stable angina pectoris (P<0.001).

Table 3. Serial Changes in Laboratory Data for Healthy Controls (n=6)

	Baseline		6 months	
	Preprandial	Postprandial	Preprandial	Postprandial
BMI	22.1±2.6		21.7±2.2	
TC (mg/dl)	176.8±18.9	173.0±22.3	176.8±32.8	171.0±31.0
HDL-C (mg/dl)	55.0±6.5	54.2±6.9	59.5±5.4	59.2±6.1
LDL-C (mg/dl)	97.7±21.2	94.7±21.7	91.7±28.9	89.5±30.0
LDL-C/HDL-C	1.82±0.53	1.79±0.56	1.54±0.49*	1.52±0.53*,#
TG (mg/dl)	81.7±38.9	155.8±52.1**	75.3±21.1	114.4±45.6#
RLP-C (mg/dl)	3.1±0.8	5.8±3.2**	4.6±2.3	5.6±1.4**
FFA (mmol/L)	0.46±0.11	0.36±0.14	0.41±0.21	0.24±0.10
Adiponectin (μg/ml)	10.55±1.65		10.65±1.72	
Plasma glucose (mg/dl)	102.2±8.2	112.3±24.4	95.0±9.1	96.0±15.4
IRI (μU/ml)	6.6±2.6	30.0±10.3**	5.0±2.5	25.9±10.1**
hsCRP (mg/L)	1.00±0.80	1.30±0.80	1.20±0.90	1.30±1.00
HbA_{1c} (%)	5.0±0.1		4.9±0.1	
Hematocrit	41.5±2.3	40.6±3.3	42.4±4.8	41.0±3.6
Blood passage time (s)	44.7±8.1	42.9±1.8	42.4±4.8	42.1±3.6
Basal FBF	5.1±0.4	5.9±1.4	5.2±0.6	6.6±1.9
FBF after NTG s.l. ($ml \cdot min^{-1} \cdot 100ml\ tissue^{-1}$)		7.7±1.5		7.8±1.8

*P<0.05 vs preprandial baseline data, **P<0.01 vs preprandial baseline data, #P<0.05 vs postprandial baseline data, ##P<0.01 vs postprandial baseline data, $P<0.05 vs preprandial data at 6 months.
Abbreviations as in Table 1.

HDL-C, TG, RLP-C and HbA_{1c} were significantly higher and levels of HDL-C and adiponectin significantly lower in the CAD patients than in healthy controls (**Table 1**). Serum levels of free fatty acids were not different. Maximum FBF during hyperemia in the baseline fasting phase was significantly lower in CAD patients ($28.8\pm8.2\ ml \cdot min^{-1} \cdot 100\ ml\ tissue^{-1}$) than in control subjects ($36.7\pm4.6\ ml \cdot min^{-1} \cdot 100\ ml\ tissue^{-1}$; P=0.040). No differences were seen at baseline in blood passage time through the microchannels.

At 2 h after taking the test meal, serum levels of TG, insulin and RLP-C were significantly increased from fasting levels in both groups, particularly in the CAD patients, and plasma glucose levels were increased only in CAD patients. Serum levels of free fatty acids remained unchanged at 2 h after the test meal. Basal FBF was slightly but significantly increased after the test meal in the CAD group (**Table 2**). Maximum FBF during hyperemia (**Figure 1**) remained unchanged in each group when compared with the fasting data. FBF after NTG administration tended to be decreased in the CAD patients as compared with healthy controls (**Table 1**). In the CAD patients, urinary 8-OHdG levels were significantly increased in the postprandial phase over fasting levels (P<0.01).

After 6-month pitavastatin treatment, serum levels of TC, LDL-C, LDL-C/HDL-C, TG and RLP-C, both fasting and postprandial, were significantly decreased while HDL-C and adiponectin levels were significantly increased compared with baseline data in the CAD patients (**Table 2**). The number of CAD patients with LDL-C >100 mg/dl decreased from 16 (100%) to 2 (13%, P<0.001 vs baseline), with TG >150 mg/dl decreased from 8 (100%) to 2 (13%, P<0.05 vs baseline), with HDL-C <40 mg/dl decreased from 10 (63%) to 0 (0%, P<0.001), and with LDL-C/HDL-C >2.0 decreased from 16 (100%) to 5 (31%, P<0.001 vs baseline).

Both fasting and postprandial maximum FBF during hyperemia were significantly increased after 6-month pitavastatin treatment (fasting, from 28.9 ± 8.2 to $36.3\pm11.5\ ml \cdot min^{-1} \cdot 100\ ml\ tissue^{-1}$, postprandial, from 31.5 ± 9.1 to $39.3\pm11.5\ ml \cdot min^{-1} \cdot 100\ ml\ tissue^{-1}$, P<0.05 vs baseline each phase data) (**Figure 1**). Fasting and postprandial maximum FBF during hyperemia were unchanged from baseline at 6 months in the control subjects. FBF after sublingual NTG administration was unchanged in both groups. Postprandial urinary 8-OHdG levels in CAD patients decreased significantly from 16.9 ± 7.6 to $6.7\pm3.8\ ng \cdot ml^{-1} \cdot creatinine^{-1}$ (P<0.001) after 6-month pitavastatin treatment (**Figure 2**). No significant correlation was seen between changes in reactive hyperemia and those in lipid profile or 8-OHdG in each prandial phase according to single logistic regression analysis (data not shown). Fasting and postprandial blood passage time through the microchannels in both groups showed no significant change after the 6-month period (**Tables 2,3**).

Discussion

The present study provides the first evidence that in patients with stable CAD, 6-month pitavastatin treatment improved postprandial endothelium-dependent vasodilatation in forearm resistance vessels to the same level as in healthy subjects, and was associated with decreased postprandial oxidative stress, TG, RLP-C and LDL-C levels, and increased adiponectin and HDL-C.

Fasting and Postprandial Endothelial Function in CAD

Baseline FBF data in response to reactive hyperemia, suggesting impairment of endothelial cells in the peripheral arteries of the patients with CAD, were consistent with previous reports.[13–15,25] In the CAD patient group, 56% had type 2 DM, which might have contributed to the impaired vasodilatory response to reactive hyperemia.[26] In general, insulin upregulates endothelial nitric oxide synthase (eNOS) via the phosphatidylinositol 3-kinase/Akt signaling pathway,[27] but this cascade is impaired in patients with DM.[28] In an animal model of insulin resistance, eNOS activity is reduced because of decreased levels of tetrahydrobiopterin, a coenzyme of eNOS, and both eNOS and NAD(P)H oxidase induce excessive production of reactive oxygen species, leading to endothelial dysfunction.[28]

Despite the postprandial metabolic changes, maximum FBF in response to reactive hyperemia was not significantly changed in either group after intake of the test meal,

especially in the CAD group exposed to oxidative stress. The present data are in line with some previous reports[17,18,25] but not all[5,7,8,13,14] Discrepancies regarding results for postprandial endothelial function may be explained as follows. First, our test meal represented carbohydrate loading (60 g) plus moderate fat intake and was associated with a high serum level of insulin at 2 h after the meal, leading to eNOS upregulation via the phosphatidylinositol 3-kinase/Akt signaling pathway. The increase in endothelium-derived NO production by eNOS upregulation could thus reach a balance with the increase in oxidative stress in our CAD patients. Second, the increase in basal FBF after meal (from 4.1±1.3 to 5.1±2.2 ml·min^{-1}·100 ml tissue^{-1}) might have somewhat confounded the pure postprandial vasodilatory response to reactive hyperemia.

Effects of Pitavastatin on Lipid Profile, Adiponectin and Oxidative Stress

In the present study, a therapeutic dose of pitavastatin (2 mg/day) decreased serum levels of LDL-C, TG, RLP-C and LDL-C/HDL-C in the fasting phase after 6 months, similar to previous reports[15,16] and also decreased postprandial levels of TG and RLP-C in the patients with CAD. RLP-C has been reported as closely associated with impaired endothelium-dependent vasomotor function in human coronary arteries[29,30] and predicts future coronary events in patients with CAD and type 2 DM or insulin resistance[31] Increased plasma adiponectin levels in CAD patients following pitavastatin administration are in line with previous results from patients with dyslipidemia[16] According to our knowledge, the present data may represent the first evidence that pitavastatin increases plasma adiponectin concentrations in patients with CAD. Adiponectin stimulates NO production in vascular endothelial cells[32] and suppresses the attachment of monocytes to endothelial cells[33] Increased adiponectin levels might have contributed to the improved endothelial function observed in the present study. Pravastatin has been reported to increase plasma adiponectin in association with improved glucose metabolism in patients with impaired glucose tolerance and CAD[34] However, effects of atorvastatin on plasma adiponectin in patients with type 2 DM seem to be controversial[35–37] A more recent report by Ando et al showed that both pravastatin and atorvastatin significantly increased plasma adiponectin in obese patients with dyslipidemia, but not in non-obese patients[36] According to recent observational studies, including our own, high adiponectin levels have been observed in several selected populations with heart failure[38–40] or renal dysfunction[41] and in an aged group[39] In those selected populations, accumulation of adiponectin in patients with heart failure or chronic kidney disease, unlike the general population, may reflect the malnutrition that characterizes these disease states and is thus a marker of poor prognosis[39,41] Because we excluded patients with heart failure, serum creatinine >1.3 mg/dl or age >76 years, increased adiponectin levels after pitavastatin treatment may act as a cardioprotective factor, including improvement of endothelial function.

Decreased postprandial urinary 8-OHdG levels after 6-month pitavastatin treatment in the CAD patients is consistent with previous reports[42,43] Membrane translocation of rac1 GTPase, which is required for the activation of NAD(P)H oxidase, is inhibited by statins[44] In addition, statins reduces NAD(P)H subunit p22^{phox}[45] Consequently, pitavastatin might decrease the urinary 8-OHdG levels in CAD patients.

Effects of Pitavastatin Treatment on Fasting and Postprandial Endothelial Function

Fasting and postprandial endothelial function was improved after 6 months of pitavastatin to the same extent as in healthy subjects. Matsumoto et al reported that 3-day treatment with pravastatin significantly improved endothelial function as evaluated by acetylcholine-induced increases in FBF measured by plethysmography in patients with CAD[46] However, van Etten et al have reported that intensive lipid lowering with atorvastatin (80 mg/day for 4 weeks) had no effect on NO availability in forearm resistance arteries as evaluated by serotonin-induced increases in FBF measured by plethysmography in patients with type 2 DM[47] which might be associated with smooth muscle dysfunction or refractory endothelial dysfunction in these patients. Sakabe et al showed rapid improvements in fasting endothelial function with pitavastatin in patients with hyperlipidemia compared with atorvastatin, despite similar effects on lipid profiles and fibrinolytic parameters[15]

Factors contributing to improvement in endothelial function following pitavastatin administration could include pleiotropic effects[46] such as anti-oxidation[42,43–45,48] as well as the LDL-C-lowering effect. Because no significant correlation was seen between changes in reactive hyperemia and those in LDL-C or 8-OHdG level in each prandial phase, we could not conclude which factor provides the greater contribution to the improvement of postprandial endothelial function by pitavastatin. Tamai et al have clearly shown that a reduction of total LDL-C and/or oxidized LDL-C by LDL-C apheresis, an alternative LDL-C-lowering treatment, improves endothelium-dependent vasodilatory response in patients with dyslipidemia[49] The inhibition of RhoA/Rho kinase activity[50] and the enhancement of phosphatidylinositol 3-kinase/Akt signaling pathway[51] by statins upregulate endothelial eNOS. The anti-oxidant effect of pitavastatin could improve NO bioavailability[42,43] thus contributing to improvement in endothelial function. Increases in plasma adiponectin[17] and blocking leukocyte function antigen 1[52] may also partly contribute to the improvement in endothelial function by pitavastatin.

Blood Rheology

The present data showed no difference in blood passage time between control subjects and CAD patients at baseline. Blood passage time also showed no significant changes after test meal intake in both groups. Pitavastatin treatment thus did not affect blood passage time in patients with CAD, despite several reports showing beneficial effects of statins on blood rheology[53–55] Two explanations are possible for the absence of abnormal blood rheology in the present CAD patients. First, all CAD patients took aspirin before entry and continued its administration throughout the study period. As the antiplatelet and anti-inflammatory effects of aspirin reportedly improve prognosis for patients with CAD, we did not feel that withdrawal of aspirin in CAD patients was ethical. Second, the present study did not include patients with poorly controlled DM or severe dyslipidemia (LDL-C ≥180 mg/dl), for whom hemorheological parameters are reportedly worse and who thus might be better candidates for improvement with pitavastatin[53–55]

Study Limitations

First, a randomized controlled trial in patients with CAD was not performed because of ethical concerns. Second, standard deviations for measurements were somewhat large,

because the sample size was relatively small. Third, discontinuation of aspirin would have been preferable for exploring the effects of pitavastatin on blood rheology, but as administration of aspirin has gained worldwide consensus for patients with CAD in terms of plaque stability, secondary prevention of cardiac events and improvements in prognosis, use of aspirin was continued in all patients with CAD.

In conclusion, administration of a clinical dose of pitavastatin appears effective for both fasting and postprandial endothelial dysfunction, and is associated with improved lipid profile, reduction in oxidative stress and increased adiponectin among patients with stable CAD.

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Disclosure

None.

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Regulation of voltage-gated K^+ channels by glucose metabolism in pancreatic β-cells

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Glucose metabolism

ABSTRACT

Regulation of delayed rectifier-type K^+ channels (Kv-channels) by glucose was studied in rat pancreatic β-cells. The Kv-channel current was increased in amplitudes by increasing glucose concentration from 2.8 to 16.6 mM, while it was decreased by 2.8 mM glucose in a reversible manner (down-regulation) in both perforated and conventional whole-cell modes. The current was decreased by FCCP, intrapipette 0 mM ATP or AMPPNP. Glyceraldehyde, pyruvic acid, 2-ketoisocaproic acid, and 10 mM MgATP prevented the down-regulation induced by 2.8 mM or less glucose. The residual current after treatment with Kv2.1-specific blocker, guangxitoxin-1E, was unchanged by lowering or increasing glucose concentration. We conclude that glucose metabolism regulates Kv2.1 channels in rats β-cells via altering MgATP levels.

1. Introduction

Potassium channels in pancreatic β-cells play a pivotal role in glucose-stimulated insulin secretion. The resting potential of β-cells is determined by activity of ATP-sensitive K^+ channels (K_{ATP} channel) [1–3]. Elevation of external glucose concentration depolarizes β-cell membrane by closure of K_{ATP} channels, thereby inducing bursting spike-like action potentials that are produced by orchestrated openings of voltage-dependent Ca^{2+} channels (VDCCs) and voltage-gated K^+ channels (Kv-channels). Kv channels are composed of delayed rectifier K^+ channels that are slowly activated upon depolarization [4,5] and a distinct class of Kv channels with fast and transient activation during depolarization, being defined as A-current [6,7]. The delayed rectifying current is the major component of Kv-channel currents in β-cells from human [8,9] and rodents [4,5]. Among these Kv channels, Kv2.1 is a predominant component of the delayed rectifying current identified in mammalian β-cells [7,8]. As the other delayed rectifying Kv channels, Kv3.2, Kv8.1 and Kv9.3 are reportedly present in β-cells [8] and furthermore Kv1.5 and Kv1.6 in human islets [7]. mRNAs of Kv1.4, Kv1.5, Kv2.1, Kv2.2, Kv3.1 and Kv3.2 channels were detected in INS-1 cells [10]. Apart from these channels, Kv1.4, Kv3.3, Kv3.4 and Kv4.2 show the feature of A-current [7]. Pharmacological, metabolic and hormonal regulations of Kv-channel current are thus expected to shorten or prolong action-potentials elicited by glucose stimulation, thereby influencing Ca^{2+} entry through VDCCs and eventually insulin secretion [11–16]. Recently it was reported that electrical activity of human β-cells during glucose stimulation was little influenced by Kv2.1 inhibition with a specific blocker, stromatoxin [17]. In the present report we demonstrate a novel regulation of delayed rectifier-type Kv-channel by glucose metabolism in rat pancreatic β-cells. The Kv-channel activity increases with an elevation of glucose concentration.

2. Materials and methods

Male Wistar rats were housed according to our institutional guidelines and for animal care. Approval of animal experiments by institutional committee of ethics was obtained. Islets of Langerhans were isolated by collagenase digestion from the rats aged 8–10 weeks, as previously reported [14,18]. Collected islets were dispersed into single cells and maintained in short-term culture for up to 3 days in Eagle's minimal essential medium containing 5.6 mM glucose supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin in 95% air with 5% CO_2 at 37 °C. The cells were superfused with control HEPES-Krebs-Ringer bicarbonate buffer (HKRB) solution containing 2.8 mM glucose.

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Perforated whole-cell clamped currents were recorded using a pipette solution containing nystatin (150–200 μg/ml) dissolved in 0.1% DMSO, as previously reported [14,18]. Membrane currents were recorded using an amplifier (Axopatch, 200B, Foster, CA) in a computer using pCLAMP10.2 software. The resistances of patch pipettes ranged from 3 to 5 MΩ.

For perforated whole-cell clamp, pipette solution contained (in mM): K_2SO_4 40, KCl 50, $MgCl_2$ 5, EGTA 0.5 and HEPES 10 at pH 7.2 with KOH. For conventional whole-cell clamp experiments, pipette solution contained (in mM): KCl 50, K_2SO_4 35, $MgCl_2$ 5, EGTA 11, $CaCl_2$ 1, HEPES 11 and ATP-2Na (Rosh Diagnostic, Tokyo, Japan) 5 at pH 7.2 with KOH. The HKRB solution contained (in mM): NaCl 129, $NaHCO_3$ 5.0, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.0, $MgSO_4$ 1.2 and HEPES 10 at pH 7.4. Glucose was added to this solution at required concentrations. Glycolytic intermediates: D,L-glyceraldehyde (Nacarai Tesque, Tokyo, Japan), pyruvic acid (Wako Pure Chemical Industries Ltd., Tokyo, Japan), or 2-ketoisocaproic acid (KIC, Nacarai Tesque, Tokyo, Japan) were applied from either external or internal side as indicated in text. Guangxitoxine-1E was from Peptide Institute Inc. (Tokyo, Japan). *p*-Trifluoromethoxyphenylhydrazone (FCCP) was from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Nystatin, Adenosine 5′-(β,γ-imido)triphosphate (AMPPNP), β-nicotinamide adenine dinucleotide phosphate hydrate (NADPH) and β-nicotinamide adenine dinucleotide phosphate (NADP) were from Sigma–Aldrich (Tokyo, Japan). The experiments were performed at room temperature (25 °C) or 36 °C (Fig. 3B). To identify whether the voltage-clamped cell is an insulin-producing cell, the cell was fixed with 4% paraformaldehyde after electrophysiological experiment and incubated for one hour with rabbit polyclonal anti-insulin antibody (MP Biomedicals, OH, USA) at a dilution of 1:250 at 36 °C followed by Alexa Fluor 488-labeled goat anti-rabbit IgG (Molecular Probes Inc., Eugene, USA). Negative control immunofluorescence was performed by omitting anti-insulin antibody (data not shown).

Data represent the mean ± S.E.M. Statistical analyses were performed using the Student's *t*-test or one-way ANOVA as indicated

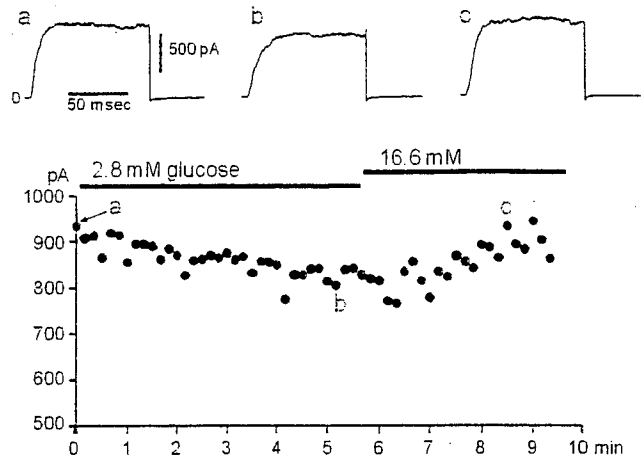

Fig. 2. Kv-channel current is down-regulated by low glucose and restored by high glucose. Amplitudes of Kv-channel current recorded from time zero in the same protocol as in Fig. 1 were plotted by measuring currents at the end of step pulses with 100 ms duration to +20 mV in the presence of 2.8 mM glucose and subsequent exposure to 16.6 mM glucose. Current traces depicted on upper panel (a–c) were obtained at the time points indicated in lower panel.

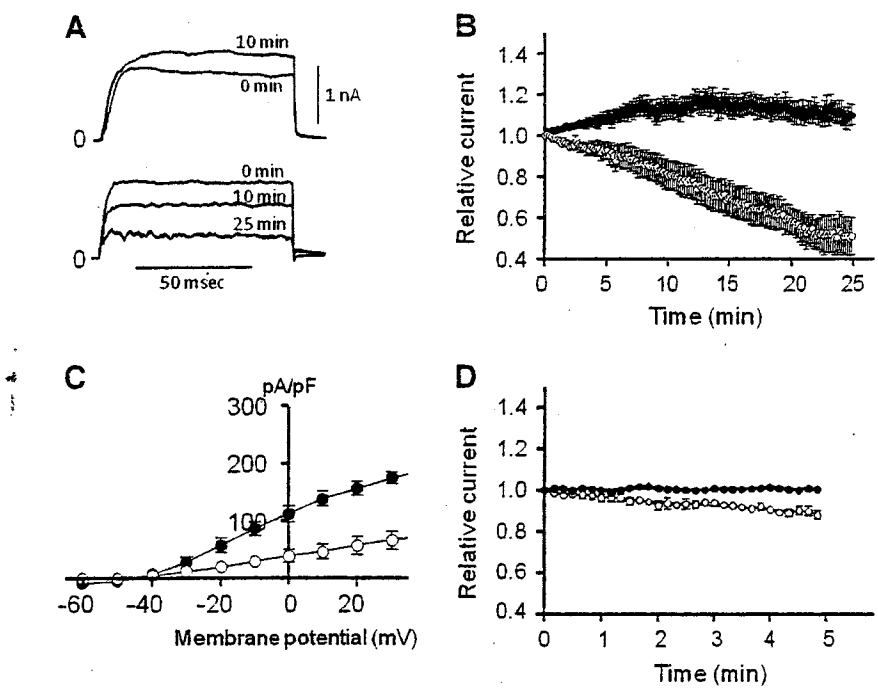

Fig. 1. Effects of glucose on the Kv-channel currents. (A) Current traces in response to depolarization to 0 mV with 100 ms duration from a holding potential of −70 mV at intervals of 10 s were recorded after formation of perforated whole-cell clamp in the presence of 2.8 mM glucose in HKRB solution. Tolbutamide at 100 μM was then added to the solution to inhibit K_{ATP} channels. At 3 min after exposure to this solution the time was reset to 0 min, the solution was changed to HKRB containing 16.6 mM glucose and recordings were initiated (upper traces). In the experiments for lower traces, the solution was continuously superfused with HKRB containing 2.8 mM glucose from time 0. Original current traces at time indicated were depicted. (B) The Kv-channel current measured at the end (a current level averaged between 90 and 99 ms) of depolarized pulses was normalized to that at time 0 and plotted against time in HKRB solution with 2.8 mM (open circles) or 16.6 mM glucose (closed circles). Data were from perforated whole-cell mode and expressed by mean ± S.E.M. (C) Current–voltage relations recorded in the presence of 2.8 mM (open symbols, $n = 6$) or 16.6 mM (closed symbols, $n = 7$) in perforated mode at 25 min. In comparison of the current densities between 2.8 and 16.6 mM glucose, P was 0.18 at −30 mV and <0.05 at more positive potentials (unpaired test). (D) Time courses of the relative Kv-channel current obtained with conventional whole-cell mode, while other protocol was the same as that in (B). Open symbols indicate data recorded from the cells exposed to 2.8 mM ($n = 4$) and closed symbols those to 11.2 mM glucose ($n = 6$).

with a software package of GrafPad Prism ver. 3.02. *P*-values below 0.05 were considered statistically significant.

3. Results

3.1. Regulation of β-cell Kv-channel current by changes in glucose concentrations

After formation of the perforated whole-cell clamp mode in control HKRB solution containing 2.8 mM glucose, the solution was changed to the HKRB solution containing 100 μM tolbutamide and either 2.8 mM or 16.6 mM glucose. Current recording was then commenced and voltage-gated activation of the currents was observed as depicted in Fig. 1A. These voltage-gated currents (Kv-channel currents) were consistent with delayed rectifying K^+ currents [4,5,7,9,14,15]. Amplitudes of Kv-channel currents gradually increased with time during exposure to 16.6 mM glucose (Fig. 1A, upper panel), while they decreased in the persistent presence of 2.8 mM glucose (down-regulation, lower panel). The relative Kv-channel currents decreased during continuous exposure to 2.8 mM glucose in a time-dependent fashion; by contrast a rise in external glucose concentration to 16.2 mM increased amplitudes of the current with time (Fig. 1B).

The relative Kv-channel current 2 min after exposure to 16.6 mM glucose was 1.04 ± 0.01 ($n = 9$, $P = 0.02$ vs. time 0 at membrane voltage of +20 mV, paired test). This was also significantly greater than that measured at the same time after exposure to 2.8 mM glucose, 0.94 ± 0.01 ($n = 10$, $P = 0.0002$ vs. 16.6 mM glucose at +20 mV, unpaired test). In terms of comparison of current levels after superfusion of 2.8 and 16.6 mM glucose, the Kv-channel current measured at 12 min were 111.9 ± 17.6 and 164.4 ± 16.3 pA/pF ($P < 0.05$), respectively. Current–voltage relations obtained 25 min after superfusion with 16.6 or 2.8 mM glucose showed glucose concentration-dependent increase in Kv-channel currents (Fig. 1C). Similar results were observed in conventional whole-cell mode (Fig. 1D). In perforated whole-cell mode the relative Kv-channel current was continuously decreased to 0.46 ± 0.08 at 25 min exposure time (Fig. 1B, open circles, $n = 5$), but it was 0.73 ± 0.01 (Supplementary Fig. 1, $n = 4$, $P = 0.03$) in conventional whole-cell mode at 2.8 mM glucose. We observed these decrease and increase in Kv-current in response to changes in glucose concentrations in all the cells examined as far as we successfully performed voltage clamp experiments for 20 min or more ($n = 52$). Furthermore, the cell in which the Kv-current decreased in the presence of 2.8 mM glucose in perforated mode was shown to be immunoreactive to insulin (Supplementary Fig. 2). These observations suggest that Kv channels of β-cells are regulated by changes in external glucose concentration.

3.2. Reversibility of down-regulated Kv-channel current by high glucose

The Kv-channel current that was down-regulated by continuous exposure to 2.8 mM glucose was restored to a control level by increasing the glucose concentration to 16.6 mM (Fig. 2), suggesting that down-regulation of the channel activity by low glucose

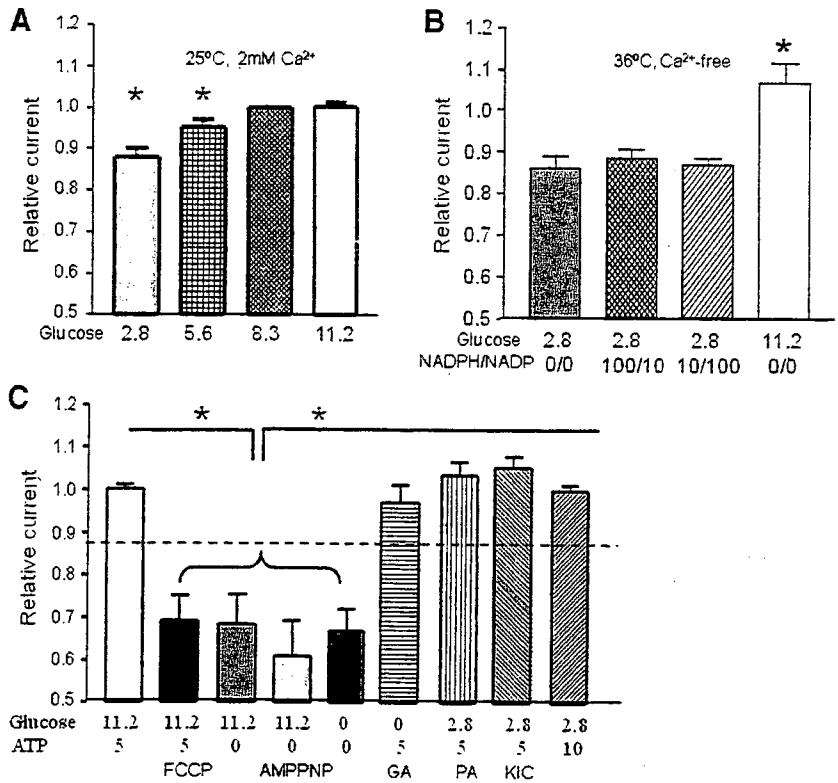

Fig. 3. Effects of glucose, temperature, ratio of cytosolic NADPH/NAD, glycolytic intermediates, metabolic inhibitions on the Kv-channel currents. All data were obtained in conventional whole-cell mode at membrane potential of +20 mV depolarized from −70 mV. (A) The relative current measured 5 min after initiation of recording with 2.8, 5.6, 8.3 or 11.2 mM glucose. $^*P < 0.05$ vs. 11.2G (unpaired test). Number of data in each bar was 4–6. (B) Relative Kv-channel current measured 5 min after initiation of each recording during exposure to 2.8 or 11.2 mM glucose in Ca^{2+}-free HKRB solution at 36 °C. To test effects of NADPH/NADP ratio in the pipette solution on Kv-channel current, 100 μM NADPH and 10 μM NADP (100/10) or 10 μM NADPH and 100 μM NADP (10/100) were included in standard pipette solution, which resulted in the relative currents of 0.89 ± 0.02 ($n = 7$) and 0.87 ± 0.02 ($n = 11$), respectively. $^*P < 0.05$ vs. the other bars by ANOVA. (C) Relative Kv-channel currents measured 5 min after initiation of each recording during exposure to 1 μM FCCP, 5 mM AMPPNP or glycolytic intermediates: 11.2 mM glyceraldehyde (GA), 10 mM pyruvic acid (PA) and 10 mM 2-ketoisocaproic acid (KIC) in the presence of different concentrations of glucose and ATP. Number of data in each bar was 5–10. Glucose and intrapipette ATP concentrations were indicated in mM in the graph. Dotted line indicated a level of the relative current at 2.8 mM glucose with 5 mM ATP as in (A). $^*P < 0.05$ by ANOVA. Data were obtained at the membrane potential of +20 mV.

may be physiological but not due to cytotoxicity induced by insufficient energy supply. We observed similar results in 3 other experiments.

3.3. Down-regulation of Kv channels is independent of temperature, external Ca^{2+} and changes in cytosolic NADPH/NADP ratio

Relative Kv-channel currents at 5 min after exposure to solutions containing varying glucose concentrations were 0.88 ± 0.02 ($n = 4$) at 2.8 mM glucose, 0.95 ± 0.02 ($n = 4$) at 5.6 mM, 1.00 ± 0.001 ($n = 4$) at 8.3 mM and 1.00 ± 0.01 ($n = 6$) at 11.2 mM (Fig. 3A). These changes in Kv-current amplitude in response to glucose were further explored in terms of temperature- and extracellular Ca^{2+}-dependence (Fig. 3B). Relative Kv-channel currents were 0.86 ± 0.03 ($n = 7$) at 2.8 mM glucose (36 °C and in Ca^{2+}-free HKRB; $P = 0.65$ vs. 2.8G in Fig. 3A: 25 °C and 2 mM Ca^{2+}). Under the same condition, relative Kv-channel currents were 1.07 ± 0.05 by continuous exposure to 11.2 mM glucose for 5 min ($n = 6$, $P = 0.003$ vs. 2.8 mM glucose in Fig. 3B). Kv2.1 channels in β-cells are also thought to be regulated by the cytosolic NADPH/NADP ratio [13]. We compared effects of different ratio of cytosolic 100:10 or 10:100 NADPH/NADP in whole-cell mode on Kv-channel current during continuous exposure to 2.8 mM glucose at 36 °C and Ca^{2+}-free in HKRB solution. The relative currents after 5 min exposure to 2.8 mM glucose were not altered by these different ratios of NADPH/NADP.

3.4. MgATP mediates the down-regulation of Kv channels

In the presence of FCCP, an uncoupler of electron transport of mitochondria [19], relative Kv-channel current measured at 5 min after exposure decreased to 0.69 ± 0.06 despite the presence of 11.2 mM glucose in HKRB solution ($P < 0.002$ vs. 11.2 mM glucose, open bar, $n = 8$, Fig. 3C). Glycolytic intermediates: 11.2 mM glyceraldehyde (GA; bath application), 10 mM pyruvic acid (PA; intrapipette use) and 10 mM 2-ketoisocaproic acid (KIC; intrapipette use) were tested at 0 or 2.8 mM glucose and all these prevented the low glucose-induced down-regulation of the Kv-channel currents. The relative Kv currents with 2.8 mM glucose and 5 mM ATP, indicated as a dotted line in Fig. 3C, were lower than those observed with GA ($P = 0.029$), PA ($P = 0.004$) and KIC ($P = 0.002$). Likewise, increase in MgATP to 10 mM in the pipette also prevented the down-regulation of Kv-channel current ($P = 0.001$). Omission of MgATP in the pipette with or without 11.2 mM glucose and non-hydrolysable ATP analogue, AMPPNP mimicked the effects of FCCP.

3.5. Kv2.1 channels as the major component of the down-regulation

The current was substantially reduced in the presence of a Kv2.1-specific inhibitor [20,21], guangxitoxin-1E (Fig. 4A). The current that remained after guangxitoxin-1E treatment exhibited a fast activating and slowly decaying kinetics, the properties known for A-current [6,9] and was insensitive to the change in glucose concentration to 11.2 or 2.8 mM (Fig. 4B). These results suggested that the component down-regulated by prolonged exposure to 2.8 mM glucose was Kv2.1 channel current.

3.6. Changes in activation and inactivation kinetics during down-regulation of Kv channels

We examined whether activation and inactivation kinetics of the Kv2.1 channels after down-regulation were influenced. We observed a leftward shift of the half-maximal conductance ($G_{1/2}$) in the conductance–voltage (G–V) relations from −7.5 ± 1.2 mV ($n = 5$) to −16.6 ± 3.0 mV ($n = 4$) after down-regulation in the continuous presence of 2.8 mM glucose (Fig. 5A). The half-maximal effect for membrane potential ($V_{i1/2}$) of steady-state inactivation was −36.9 ± 2.6 mV in control and −39.6 ± 2.6 mV after 5 min exposure to 2.8 mM glucose ($n = 4$, Fig. 5B). The reduction of Kv-channel current at low glucose was use-dependent (Fig. 5C). Voltage pulses to +20 mV at intervals of 10 s for 3 min followed by 5 min pause at a holding potential of −70 mV were repeated during the continuous exposure to 2.8 mM glucose. The current amplitudes during repetitive pulses decreased with time and partially restored after the pauses. This use-dependent down-regulation was not observed in the presence of 11.2 mM glucose (Fig. 5D). The relative currents were increased during exposure to higher glucose concentration.

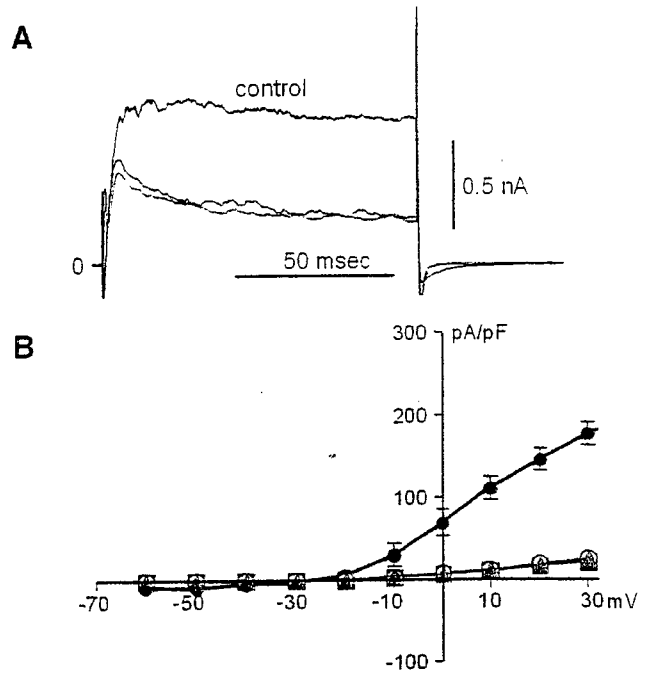

Fig. 4. Selective regulation of Kv2.1 channels by glucose metabolism. (A) After adding tolbutamide to HKRB solution, the current traces at +20 mV (control) and those in the presence of 30 nM guangxitoxin-1E before (black) and at 5 min after (red line) exposure to 2.8 mM glucose were shown. (B) Current densities in 2.8 mM glucose (control, closed circles) were decreased by exposure to 30 nM guangxitoxine-1E (open circles). The current–voltage relations after the guangxitoxin-1E were not influenced by 5 min exposure to low glucose (2.8 mM; closed squares) and high glucose (11.2 mM; closed triangles). These three current–voltage relations were completely overlapped. Number of data in each group was 4.

4. Discussion

In the present report we demonstrated a regulation of the Kv-channel current by glucose metabolism in pancreatic β-cells. The Kv-channel current increased in response to elevation of glucose concentration in a physiological range and it decreased upon prolonged exposure to low glucose in both perforated and conventional whole-cell clamp modes (down-regulation of the channel current). These glucose effects may be due to modulation of the channel activity by glucose metabolism, whereas changes in NADPH/NADP ratio, temperature or extracellular Ca^{2+} concentration did not influence time-course and magnitude of down-regulation of the channel current. Increase of osmotic pressure by an addition of 8.4 mM sucrose in the presence of 2.8 mM glucose did not mimic the effect of 11.2 mM glucose on the Kv-channel activity; the relative Kv-channel current 5 min after exposure was 0.70 ± 0.03 in the former ($n = 4$, $P < 0.0001$; unpaired test vs. 11.2 mM glucose). Thus, glucose metabolism but not osmotic pres-

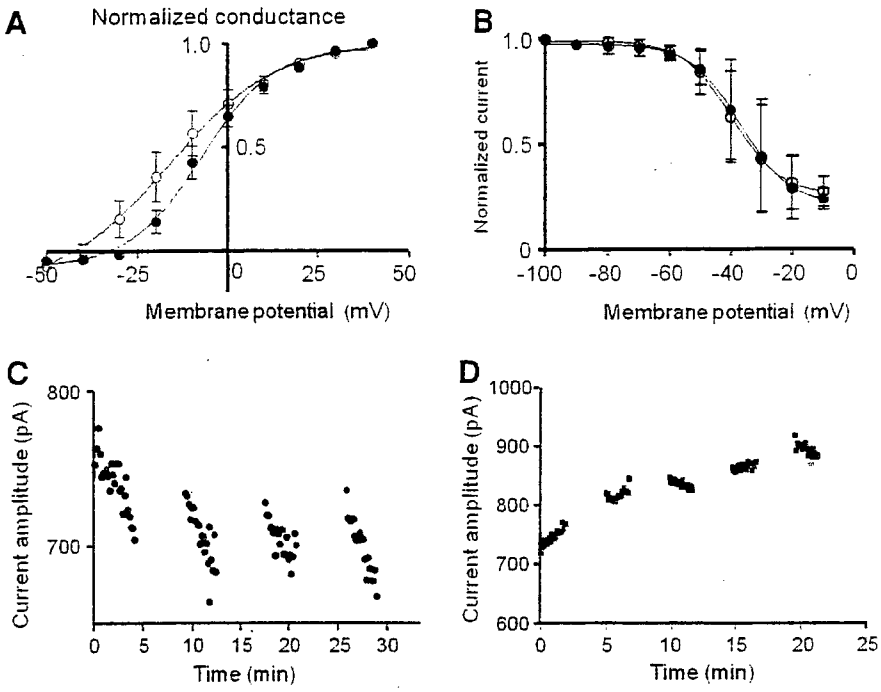

Fig. 5. (A) Cord conductance–voltage (G–V) relations before and after down-regulation of Kv channels. Membrane potential was depolarized to +50 mV in 10 mV step with an interval of 20 s between pulses from a holding potential of −70 mV. Cord conductances were calculated by dividing the current amplitudes measured at the end of each test pulse with a difference between the test potential and E_K that was calculated from the Nernst equation (−80.7 mV in our solutions). Cord conductances were normalized to that obtained at +50 mV and plotted against membrane potentials. The Boltzmann equation was used to fit these data. Closed and open circles were data before and after the down-regulation that was produced by exposure to 2.8 mM glucose for 5 min, respectively. (B) Steady-state inactivation curves before (closed circles) and after (open circles) the down-regulation of Kv-channel current. In terms of voltage protocol, prepulses of various potentials between −100 and −10 mV in 10 mV step with a long duration (10 s) were applied to inactivate the channels, and followed by a short hyperpolarization (−100 mV) of 10 ms with subsequent depolarizing pulse to +20 mV of 100 ms to measure a relative level of steady-state inactivation. Normalized relative currents were plotted and Boltzmann equation was also used to fit the data. (C) Use-dependent down-regulation of Kv channels at low glucose. The Kv-channel currents were measured at the end of test pulses to +20 mV at 10 s intervals with the same voltage protocol as used in previous figures and plotted against time. The depolarized pulses were applied for 3 min and paused for 5 min. These intermittent stimulations and pause were repeated as illustrated. The cells were continuously superfused with HKRB solution containing 2.8 mM glucose throughout the experiment. The down-regulation was partially restored after pauses. (D) Kv-channel current were increased by exposure to 11.2 mM glucose. Voltage protocol for intermittent stimulations and pauses was same as in (C) except for continuous exposure to high glucose. Date from whole-cell mode and 25 °C.

sure is crucial in Kv-channel regulations. External application of glyceraldehyde and intracellularly dialysed intermediates, pyruvic acid or KIC, mimicked the effect of high glucose and prevented the channel down-regulation in the presence or absence of 2.8 mM glucose. Application of KIC to β-cell at 0 mM glucose-stimulated insulin secretion by increasing the intracellular ATP levels [22]. Glyceraldehyde also was used as an intermediate product of glycolysis [23]. Both of the intermediates inhibited openings of K_{ATP} channels activated under conditions of metabolic inhibition in pancreatic β-cells [18]. Thus, glycolysis as well as mitochondrial metabolism may contribute to maintenance of the Kv-channel activity. Increase in internal MgATP concentration to 10 mM in whole-cell pipette solution also stabilized the channel activity, while FCCP and 0 mM ATP with or without 11.2 mM glucose produced down-regulation of the Kv channel. Non-hydrolysable analogue of ATP, AMPPNP, did not mimic the ability of high glucose to prevent the Kv-channel down-regulation (Fig. 3C). These results suggest that MgATP plays a pivotal role in maintaining the activity of the channel.

This Kv-channel regulation by glucose metabolism was specific for Kv2.1 channels but not for A-current, because the residual channel current after inhibition of Kv-channels by guangxitoxin-1E remained unaffected during superfusion with 2.8 or 11.2 mM glucose. Guangxitoxin-1E is a blocker selective to Kv2.1 and Kv2.2 channels with a half-maximal concentration of ~1 nM [20,21]. In rat β-cells, Kv2.1 but not Kv2.2 channels are reportedly present [7]. Accordingly, Kv2.1 channel appears to be the primary Kv-channel subtype that is targeted by glucose.

Direct phosphorylation of Kv2.1 channels influences channel kinetics [24–26]. Kv2.1 channels are highly phosphorylated and graded dephosphorylation shifted $G_{1/2}$ in the G–V relations toward more negative potentials as compared to that in control [26]. In the present paper, we observed similar leftward shift of $G_{1/2}$ from −7.5 ± 1.2 to −16.6 ± 3.0 mV after down-regulation in the continuous presence of 2.8 mM glucose (Fig. 5A). $V_{i1/2}$ of steady-state inactivation was −36.9 ± 2.6 mV in control and −39.6 ± 2.6 mV after 5 min exposure to 2.8 mM glucose (Fig. 5B). Dephosphorylated Kv2.1 channels reportedly showed a shift of $V_{i1/2}$ toward negative potential [26]. Although this small shift of the $V_{i1/2}$ was insignificant, dephosphorylation of Kv-channels might be in part involved in the down-regulation of Kv-channel currents during exposure to low glucose. The ineffectiveness of low-glucose exposure on inactivation kinetics suggests that changes in one or more of the following parameters: maximum open probability of Kv channels, availability of number of the channels and recovery from inactivation between pulses may be involved. ATP depletion and dephosphorylation as a consequence of low MgATP levels as substrate may mechanistically contribute to down-regulation of Kv channels.

We also found that intracellular energy metabolism is not chemically clamped in conventional whole-cell mode and influenced by changes in external metabolic environment such as glucose concentrations. Reduction of the Kv-channel currents by 2.8 mM glucose in the whole-cell mode was less pronounced than that in the perforated whole-cell mode (Fig. 1B and Supplementary Fig. 1). Therefore, it is suggested that cytoplasmic ATP levels can

change in the conventional whole-cell mode but to a lesser extent than the perforated whole-cell mode, because ATP is supplied continuously through pipettes in whole-cell mode.

Time required until initiation of recordings after bringing the cells out of the CO_2 incubator was 21.0 ± 3.7 min (n = 5) in perforated whole-cell mode and 11.7 ± 1.4 min (n = 35) in conventional whole-cell clamp mode. The current density at time 0 for recordings was 156.7 ± 18.7 pA/pF (n = 10) in the perforated mode and 166.6 ± 17.3 pA/pF (n = 10, P > 0.05) in the conventional mode. These results suggest that the Kv-channel activity is stable unless the membrane is voltage-clamped to depolarized potentials. In fact, we observed that Kv-channel current was down-regulated in a use-dependent manner and partially restored from the down-regulation after pauses without depolarized pulse at low glucose (Fig. 5C) and up-regulated (increase in the current) at high glucose (Fig. 5D).

Our results that glucose metabolism enhances the Kv-channel activity may suggest a negative-feedback regulation of action potentials in response to glucose stimulation. This may reduce an excess entry of Ca^{2+} ions into the cell during action-potential bursting. Because there is no specific opener for Kv2.1 channels yet, we cannot evaluate effects of increase in Kv-channel current at high glucose on insulin secretion. Time required for 50% repolarization of action potential in β-cells was short during glucose stimulated electrical firings (48.8 ± 4.0 ms, n = 6). The time constant for Kv-channel activation on depolarized pulses (6.6 ± 1.7 ms at −10 mV, n = 5) was fast enough to influence action-potential durations. Thus, even in these short action-potential durations, both electrical activity and insulin secretion are reportedly influenced by Kv-channel blocker, guangxitoxin-1E [20]. We have previously observed that ghrelin, a gastric-derived hormone with potential stimulatory effects on feeding and growth hormone release, inhibits glucose-stimulated insulin secretion from pancreatic β-cells by an involvement of increase in Kv-channel current [14,15]. Whether insulin secretion is negatively regulated by an increase in Kv-channel availability at high glucose remains to be elucidated.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.05.050.

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An Increase in Serum Retinol-Binding Protein 4 in the Type 2 Diabetic Subjects with Nephropathy

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Abstract. The present study was undertaken to determine retinol-binding protein 4 (RBP4) levels in subjects with diabetic nephropathy. A total of 149 type 2 diabetic subjects and 19 control subjects were enrolled. Serum levels of RBP4 were measured by a method of ELISA. Serum RBP4 levels were significantly greater in the subjects with type 2 diabetes mellitus than the controls (70.5 ± 35.3 vs. 40.1 ± 13.0 μg/ml, mean ± SD, $p<0.01$). Serum RBP4 levels were gradually increased according to the progression of diabetic nephropathy (p value in trend test: <0.001). Its elevation was significantly greater in the diabetic subjects with stages 1, 3B and 4 than the control subjects (Stage 1: 64.6 ± 29.7, Stage 3B: 123.3 ± 71.8, Stage 4: 91.4 ± 33.8 vs. Control: 40.1 ± 13.0 μg/ml, $p<0.01$). Similar results were obtained in the subjects based on the amount of albuminuria (Normo-: 64.6 ± 29.7, Micro-: 63.7 ± 29.4, and Marcoalbuminuria: 90.3 ± 44.6 μg/ml, $p <0.001$). Serum RBP4 levels had a positive correlation with serum creatinine levels($r = 0.377$, $p<0.001$), and a negative correlation with 1/creatinine ($r = -0.420$, $p<0.001$). Also, there was a negative correlation between serum RBP4 and the estimated glomerular filtration rate($r = -0.436$, $p<0.001$). Multiple regression analysis showed that estimated glomerular filtration rate was an independent determinant for increased serum RBP4 levels. There was no difference in serum RBP4 levels between the advanced nephropathy with and without macrovascular diseases. These results indicate an increase in serum RBP4 levels in the type 2 diabetic subjects, particularly complicated with advanced renal impairment.

Key words: RBP4, Diabetes mellitus, Diabetic nephropathy, Albuminuria, estimated GFR

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RETINOL-BINDING protein 4 (RBP4) is a small protein (molecular weight approximately 21 kDa), which is synthesized mainly in hepatocytes. RBP4 is also expressed in skeletal muscles and white adipose tissues, that are sensitive to insulin [1]. RBP4 binds to retinol and transthyretin, and delivers to the tissues [2, 3]. It is reported that RBP4 is increased in plasma of subjects with obesity, impaired glucose tolerance and diabetes mellitus [4, 5]. Yang *et al.* [6] showed that RBP4 is closely linked to insulin resistance in obesity and type 2 diabetes. Alterations in RBP4-transthyretin binding contribute to elevated serum RBP4 levels in insulin-resistant states [7]. However, there are several controversial results regarding insulin resistance [8–13]. In diabetic nephropathy plasma RBP4 levels are further elevated in diabetic subjects with microalbuminuria [14–16].

Diabetic nephropathy is the major microvascular complication of diabetes mellitus, which could progressively develop renal impairment. Endothelial dysfunction may be responsible for accelerated atherosclerosis in chronic kidney diseases. Damaged endothelial cells secrete cytokines and growth factors, and they accumulate into the subendothelial space of the injured region, thus promoting atherogenic change [17–22]. Adipose tissues also produce and secrete many adipokines, including adiponectin, leptin, tumor necrosis factor α, RBP4 and others. Among them, they group into 2 types regarding inflammatory and atherogenic effects. It is well known that tumor necro-

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