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Clinical Significance and Reproducibility of New Arterial Distensibility Index

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Background The brachial–ankle pulse wave velocity (baPWV) is used to evaluate the degree of atherosclerosis and arterial distensibility, but its major limitation is that it is affected by changes in blood pressure (BP) during measurement. Recently, a new atherosclerotic index, the cardio-ankle vascular index (CAVI), has been developed by measuring PWV and BP. CAVI is adjusted for BP based on the stiffness parameter β and should measure arterial stiffness independent of BP. The purpose of this study was to evaluate the validity of CAVI compared with baPWV, the reproducibility of the measurement of CAVI, and the effect of BP changes on CAVI and baPWV.

Methods and Results One thousand and thirty-three consecutive subjects undergoing health checkups were studied. CAVI was automatically calculated from the pulse volume record, BP, and the vascular length from heart to ankle. In this general population, both baPWV and CAVI demonstrated a positive correlation with age and systolic BP (SBP). CAVI showed a weaker correlation with SBP than baPWV. The measurement of CAVI demonstrated good reproducibility and was not affected by the increase in BP during measurement.

Conclusions CAVI is a useful index of arterial distensibility and is not influenced by BP changes during measurement. (*Circ J* 2007; **71:** 89–94)

Key Words: Blood pressure; Brachial–ankle pulse wave velocity; Cardio-ankle vascular index

Pulse wave velocity (PWV) has been used as a noninvasive clinical index of aortic stiffness[1] An elevated carotid–femoral PWV (cfPWV) has been reported to predict cardiovascular events and all-cause mortality in hypertensive patients and in the general population[2–5] Although conventional techniques for measuring cfPWV are noninvasive, a femoral artery transducer carefully adjusted to obtain an accurate pulse wave is required, which increases psychological stress. Also, such sophisticated and complex techniques are inconvenient, particularly in large clinical trials.

A simple, noninvasive, and automatic method of measuring the brachial–ankle PWV (baPWV) has been developed and a close correlation between baPWV and aortic PWV has been reported[6] BaPWV has also been shown to be a predictor for the presence of coronary artery disease[7] and the prognosis of acute coronary syndrome[8] Furthermore, baPWV correlates with abdominal aortic calcification[9] and carotid intima–media thickness[10] We have previously reported that baPWV correlates with age in healthy subjects, suggesting that it reflects age-related changes in vascular stiffness[11] Along with age, blood pressure (BP) is a major determinant of baPWV[12,13] It is a problem that baPWV is affected by changes in BP during measurement. Recently, a new atherosclerotic index, the cardio-ankle vascular index (CAVI), was developed by measuring baPWV and BP[13,14] CAVI is adjusted for BP based on a stiffness parameter β and is believed to measure arterial stiffness independent of BP.

We conducted this 3-part study to evaluate the validity of CAVI, comparing it with baPWV (Study 1), the reproducibility of the measurement of CAVI (Study 2), and the effect of BP changes on CAVI and baPWV (Study 3).

Methods

Study 1: Comparison of CAVI and baPWV

The study group consisted of 1,033 consecutive subjects (567 men, 466 women, mean age: 50±15 years, range: 18–82) undergoing routine health checkups at JA Kagoshima Kouseiren Medical Health Care Center. On the basis of personal interviews, 114 subjects were receiving treatment for hypertension, 11 were receiving treatment for diabetes mellitus, and 37 were receiving treatment for hyperlipidemia; 14 subjects had a history of ischemic heart disease, and 10 had a history of stroke. Information on smoking history was obtained by means of a self-administered questionnaire. Blood samples were taken after the subjects had fasted overnight. Serum concentrations of total cholesterol (TC), triglyceride (TG), and high-density lipoprotein-cholesterol (HDL-C) were measured by standard laboratory procedures. Low-density lipoprotein-cholesterol (LDL-C) was calculated by the Friedwald equation. Twelve subjects with a serum TG concentration of 400 mg/dl or higher were excluded from calculation of LDL-C.

CAVI and baPWV were measured using a Vasera VS-1000 (Fukuda Denshi, Tokyo, Japan). Cuffs were applied to

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Table 1 Characteristics of Subjects

Variable	Men (n=567)	Women (n=466)	All subjects (n=1,033)
Age (years)	50±15	49±15	50±15
BMI (kg/m²)	23.5±3.2	21.8±3.2*	22.8±3.3
TC (mg/dl)	204±33	207±31	205±32
TG (mg/dl)	128±82	88±54*	110±73
HDL-C (mg/dl)	54±14	63±15*	58±15
LDL-C (mg/dl)	124±31	126±29	125±30
FBS (mg/dl)	106±21	98±19*	103±21
Hemoglobin A1c (%)	5.3±0.7	5.2±0.5**	5.2±0.6
BUN (mg/dl)	16.2±4.1	14.5±4.0*	15.4±4.2
Creatinine (mg/dl)	0.8±0.2	0.6±0.1*	0.7±0.2
UA (mg/dl)	5.9±1.2	4.2±1.0*	5.2±1.4
HR (beats/min)	70±12	70±11	70±12
SBP (mmHg)	129±15	122±18*	126±17
DBP (mmHg)	83±11	77±12*	81±12
MBP (mmHg)	100±14	94±15*	98±15
PP (mmHg)	46±9	45±11	45±10
Smoking history (%)	60	4*	36
baPWV (cm/s)	1,383±290	1,318±272*	1,354±284
CAVI	8.5±1.5	8.1±1.3*	8.3±1.4

BMI, body mass index; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; FBS, fasting blood sugar; BUN, blood urea nitrogen; UA, uric acid; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; PP, pulse pressure; baPWV, brachial-ankle pulse wave velocity; CAVI, cardio-ankle vascular index.
**p<0.05 vs men; **p<0.0001 vs men.*

the 4 extremities, and electrocardiographic electrodes were attached to the upper arm. A microphone was placed on the sternal angle for phonocardiography. The subjects rested in the supine position for 5 min. PWV was calculated by dividing the distance from the aortic valve to the ankle artery by the sum of the difference between the time the pulse waves were transmitted to the brachium and the time the same wave were transmitted to the ankle, and the time difference between the 2nd heart sound on the phonocardiogram and the notch of the brachial pulse waves[13,14] To minimize cuff inflation effects on blood flow dynamics, pulse waves were measured with cuffs inflated to lower than the diastolic BP (DBP: 50 mmHg). Then, the extremity BP was measured by oscillometry. Systolic BP (SBP), DBP and pulse pressure (PP) were obtained by measuring the BP at the right brachial artery. In this study, none of the patients had peripheral artery disease as evidenced by an ankle–brachial index of less than 0.9.

CAVI was obtained by substituting the stiffness parameter β in the following equation for determining vascular elasticity and PWV. The stiffness parameter indicates BP-independent patient-specific vascular stiffness measured by arterial ultrasound[15] The stiffness parameter β is calculated by the following:

$$\text{Stiffness parameter } \beta = \ln(Ps/Pd) \times (D+\Delta D)/\Delta D \quad (1)$$

where Ps and Pd are respectively the SBP and DBP in mmHg. D is the diameter of blood vessel and ΔD is the change of D.

PWV can be estimated by the Bramwell-Hill equation as follows:

$$(D+\Delta D)/\Delta D = 2\rho \times 1\,(Ps-Pd) \times PWV^2 \quad (2)$$

where ρ is the density of blood.

If we substitute equation (2) for equation (1), we obtain:

$$\text{Stiffness parameter } \beta = 2\rho \times 1/(Ps-Pd) \times \ln(Ps/Pd) \times PWV^2 = CAVI.$$

The CAVI can therefore be obtained from measurement of BP and PWV.

Study 2: Reproducibility of Measurement of CAVI

CAVI was measured twice for each patient within an interval of 2 weeks between the 2 measurements in 21 consecutive patients at Kagoshima University Hospital. The reproducibility of CAVI was examined by linear regression analysis of the calculated values obtained from each pair of measurements.

Study 3: Influence of BP Changes on CAVI and baPWV

In order to analyze the effect of BP changes on CAVI and baPWV, we measured these 2 indices at the 2 time points in consecutive 62 patients taking antihypertensive medicine and selected 27 patients in whom there were changes in the SBP of more than 10 mmHg. For each index, differences between the mean values at the 2 time points of measurement were analyzed by paired t-test.

The protocol used for the present study was approved by the Institutional Review Board of Kagoshima University. Informed consent was given by all volunteers and patients.

Statistical Analysis

Data are expressed as the mean±SD. Differences between mean values of 2 groups were analyzed by unpaired t-tests. In Study 3, differences between the mean values at the 2 time points of measurement were analyzed by paired t-test. The relationship between continuous variables was analyzed by linear regression analysis. The independence of the association between variables was tested with multiple regression analysis. Bland-Altman analysis was used to assess the reproducibility of CAVI measurement. Statistical analyses were performed with Stat View, version 5.0 or Prism, version 4.0, and p-values less than 0.05 were considered statistically significant.

Results

Study 1: Comparison of CAVI and baPWV

The clinical characteristics of the subjects are summarized in Table 1. There was no significant difference in age between men and women (men: 50±15 years, women: 49±15 years). The proportion of subjects with a history of smoking was significantly higher in men than in women. The differences in atherosclerotic risk factors between men and women in the general population were shown in the study subjects.

The relationships between baPWV or CAVI and age or SBP were analyzed. BaPWV and CAVI correlated positively with age in men (baPWV: r=0.675, CAVI: r=0.739, p< 0.0001, respectively) (Figs 1A,B) and in women (baPWV: r=0.752, CAVI: r=0.750, p<0.0001, respectively) (Figs 2A, B).

Because the conventional measurement of PWV is dependent on BP, the relationships of baPWV and CAVI to SBP were analyzed. BaPWV strongly correlated with BP in both men and women (Figs 1C,2C), whereas CAVI only showed a weak correlation with SBP (Figs 1D,2D).

In men, baPWV significantly correlated with TC, TG, fasting blood sugar (FBS), hemoglobin (Hb) A1c, blood urea nitrogen (BUN) and serum creatinine, and CAVI sig-

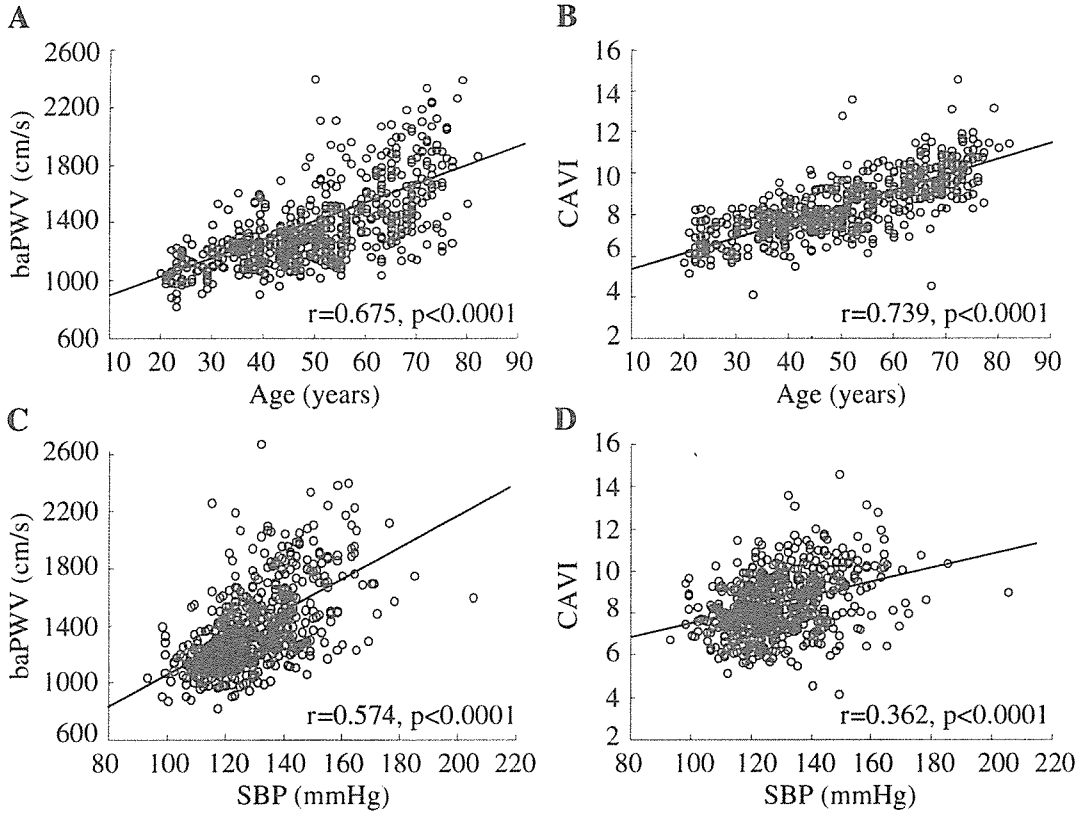

Fig 1. Relationship between baPWV and age (A), CAVI and age (B), baPWV and SBP (C), and CAVI and SBP (D) in 567 men. baPWV, brachial-ankle pulse wave velocity; CAVI, cardio-ankle vascular index; SBP, systolic blood pressure.

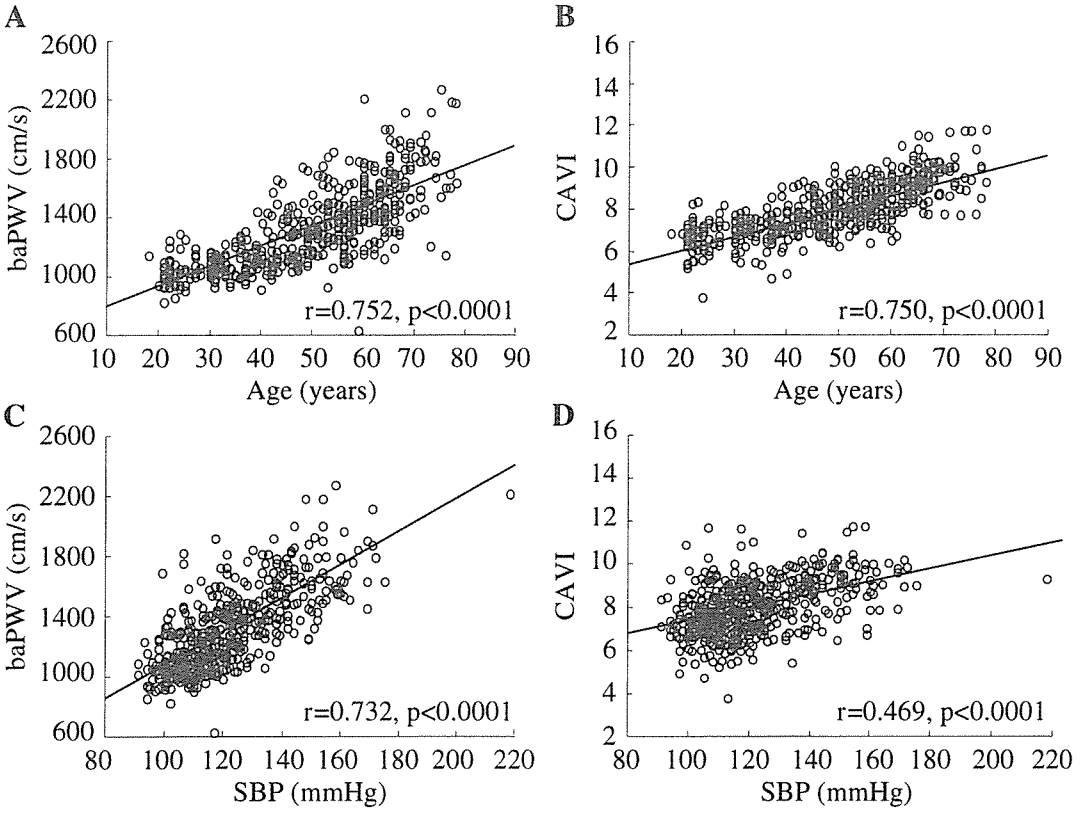

Fig 2. Relationship between baPWV and age (A), CAVI and age (B), baPWV and SBP (C), and CAVI and SBP (D) in 466 women. baPWV, brachial-ankle pulse wave velocity; CAVI, cardio-ankle vascular index; SBP, systolic blood pressure.

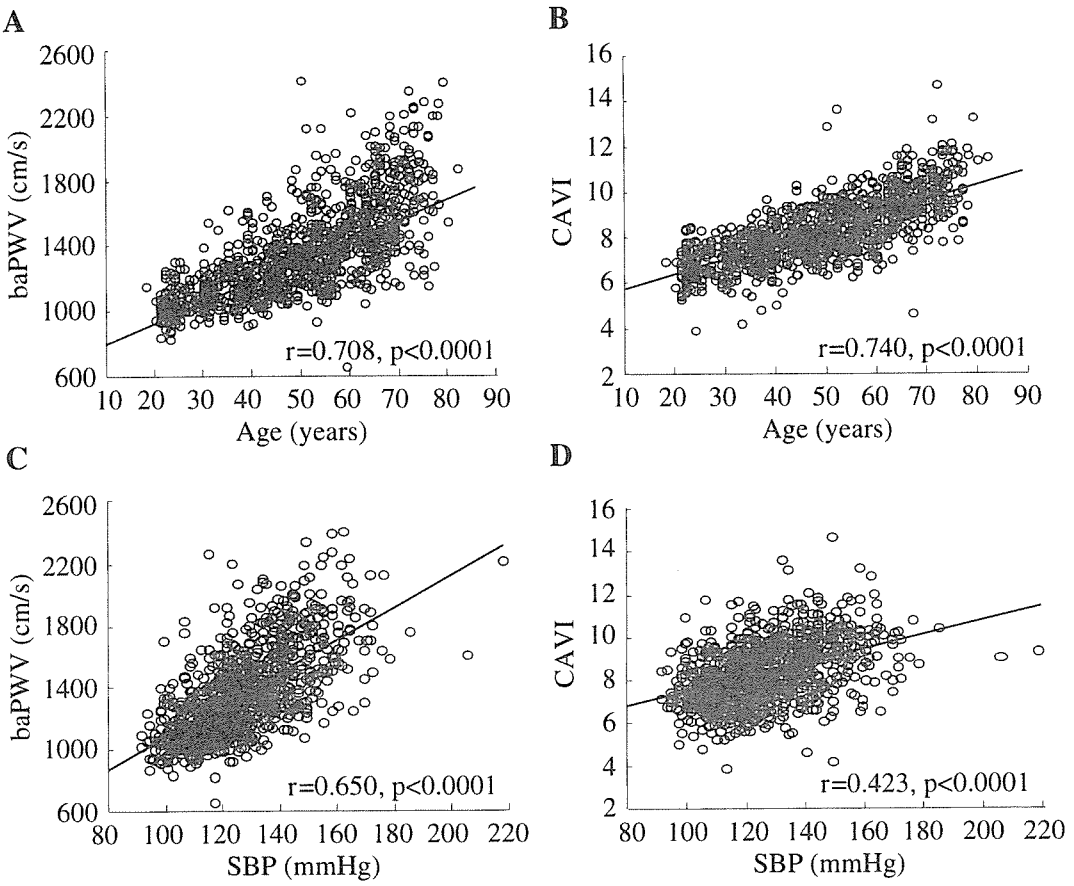

Fig 3. Relationship between baPWV and age (A), CAVI and age (B), baPWV and SBP (C), and CAVI and SBP (D) in all 1,033 subjects. baPWV, brachial-ankle pulse wave velocity; CAVI, cardio-ankle vascular index; SBP, systolic blood pressure.

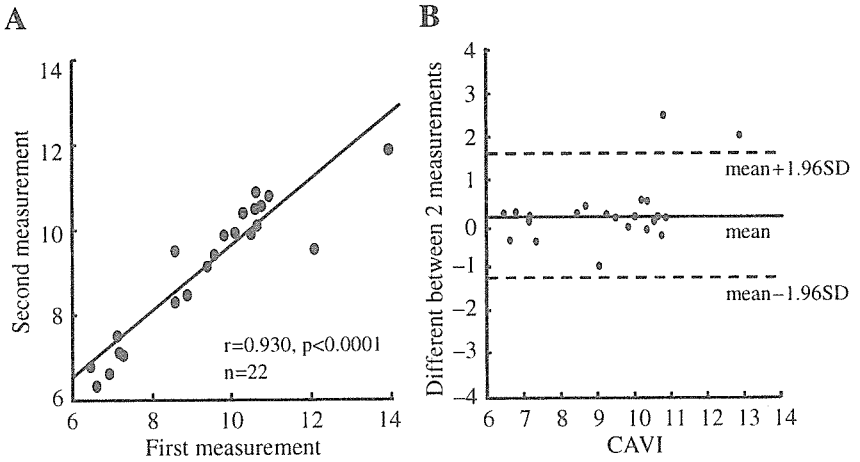

Fig 4. Reproducibility of measurements of CAVI. (A) Linear regression analysis of 2 different measurements of CAVI. (B) Bland-Altman plots of 2 different measurements of CAVI. CAVI, cardio-ankle vascular index.

nificantly correlated with FBS, HbA1c, BUN, and serum creatinine (Table 2). In the multiple regression analysis of baPWV or CAVI and other clinical variables baPWV independently correlated with age, SBP, history of smoking, TG and FBS, and CAVI independently correlated with age, SBP, TG and history of smoking (Table 3).

In women, baPWV significantly correlated with TC, TG, HDL-C, LDL-C, FBS, HbA1c, BUN, serum creatinine and uric acid, and CAVI significantly correlated with TC, TG, HDL-C, LDL-C, FBS, HbA1c, BUN and uric acid (Table 2). In the multiple regression analysis between baPWV or CAVI and other clinical variables baPWV independently correlated with age, SBP and PP, and CAVI independently correlated with age and serum creatinine (Table 3).

In all subjects, multiple regression analysis showed that baPWV independently correlated with age, TG, FBS, SBP and PP, and CAVI independently correlated with age, FBS, SBP and smoking.

Table 2 Coefficients of Correlation From Linear Regression Analysis of baPWV or CAVI and Other Variables

Variable	Men		Women		All subjects	
	baPWV	CAVI	baPWV	CAVI	baPWV	CAVI
Age	0.675*	0.739*	0.752*	0.750*	0.708*	0.740*
TC	0.143**	0.087	0.259*	0.275*	0.185*	0.153*
TG	0.120**	0.051	0.254*	0.187**	0.186*	0.135*
HDL-C	0.019	0.004	0.196*	0.169**	0.110**	0.119**
LDL-C	0.078	0.058	0.283*	0.312*	0.162*	0.156*
FBS	0.279*	0.239*	0.215*	0.196*	0.268*	0.247*
Hemoglobin A1c	0.234*	0.218*	0.183**	0.129**	0.220*	0.198*
BUN	0.181*	0.219*	0.359*	0.377*	0.275*	0.310*
Creatinine	0.136**	0.100**	0.112**	0.091	0.167*	0.175*
UA	0.029	0.040	0.218*	0.143**	0.147*	0.120**
SBP	0.574*	0.362*	0.732*	0.469*	0.650*	0.423*
DBP	0.540*	0.345*	0.691*	0.476*	0.611*	0.418*
MBP	0.617*	0.423*	0.753*	0.519*	0.682*	0.477*
PP	0.285*	0.175*	0.496*	0.283*	0.387*	0.228*

Abbreviations see in Table 1.
**p<0.0001; **p<0.05.*

Table 3 Multiple Regression Analysis of CAVI or baPWV and Other Variables

Variable	Men				Women				All subjects			
	baPWV (R^2=0.60)		CAVI (R^2=0.56)		baPWV (R^2=0.67)		CAVI (R^2=0.47)		baPWV (R^2=0.61)		CAVI (R^2=0.49)	
	Coefficient	p value	Coefficient	p value	Coefficient	p value	Coefficient	p value	Coefficient	p value	Coefficient	p value
Age	12.10	<0.0001	0.072	<0.0001	10.54	<0.0001	0.062	<0.0001	11.42	<0.0001	0.068	<0.0001
TC		NS		NS		NS		NS		NS		NS
TG	0.35	<0.05	0.001	<0.05		NS		NS	0.24	<0.05		NS
HDL-C		NS		NS		NS		NS		NS		NS
FBS	1.94	<0.05		NS		NS		NS	1.19	<0.05	0.007	<0.05
Hemoglobin A1c		NS		NS		NS		NS		NS		NS
BUN		NS		NS		NS		NS		NS		NS
Creatinine		NS		NS		NS	1.02	<0.05		NS		NS
UA		NS		NS		NS		NS		NS		NS
SBP	10.25	<0.0001	0.019	<0.001	8.72	<0.0001		NS	9.45	<0.0001	0.014	<0.001
PP		NS		NS	−2.86	<0.05		NS	−3.84	<0.01		NS
Smoking	47.31	<0.05	0.310	<0.01		NS		NS		NS	0.308	<0.01

NS, not significant. Other abbreviations see in Table 1.

Study 2: Reproducibility of Measurement of CAVI

The reproducibility of CAVI measurements was analyzed in 21 consecutive patients at Kagoshima University Hospital. CAVI was measured twice for each patient with an interval of 2 weeks. There was no significant difference in BP between the 2 measurements. Linear regression analysis showed a very strong correlation between the 2 measurements of CAVI (r=0.93, p<0.0001). In Bland-Altman plots, the mean difference of 2 measurements was 0.2, and the 95% limits of agreement (mean±1.96SD) was −1.2 and 1.7.

Study 3: Effect of BP Changes on CAVI and baPWV

To analyze the effect of BP changes on CAVI and baPWV, we measured that these 2 indices at the 2 time points in 27 patients who showed a change in SBP of more than 10 mmHg (13.3±3.2 mmHg, range 10–20 mmHg). Although baPWV increased in proportion to the increase in SBP, CAVI did not show a significant change between the 2 measurements (baPWV: 1,433±348 cm/s vs 1,489±381 cm/s, p<0.05; CAVI: 9.0±1.5 vs 9.1±1.5, not significant).

Discussion

In Study 1, both baPWV and CAVI demonstrated positive correlations with age and SBP, although CAVI showed a weaker correlation with SBP than did baPWV. In Study 2, good reproducibility of the CAVI measurements was demonstrated and in Study 3, we demonstrated that CAVI was not affected by changes in BP.

Aging causes degeneration of elastic fibers and is associated with stretching and remodeling of the arterial wall, which leads to increased collagen fibers and accumulation of smooth muscle cells. With aging, arteries progressively stiffen, dilate, and lengthen, and the arterial wall thickens[16,17] Increased arterial stiffness has been reported to lead to the age-related increase in PWV[18,19] Consistent with those previous reports, in the present study we demonstrated that CAVI, as with baPWV, significantly correlated with age.

Along with age, BP is a major determinant of PWV. PWV increases with increased stiffness of the vascular wall and increases in the internal pressure. CAVI is adjusted for BP based on the stiffness parameter β and is hypothesized to measure arterial stiffness independent of BP. In the present study, CAVI demonstrated a weaker correlation with SBP than did baPWV. Furthermore, multiple regression analysis demonstrated that SBP was not an independent determinant of CAVI in women. Shirai et al recently reported that CAVI was correlated weakly with SBP and DBP compared with baPWV in 482 hemodialysis patients[14] which is the same tendency shown in our results, which were obtained in a general population.

It is problematic that baPWV increases with increases in the BP during measurement and patients have to rest prior to measurement in order to avoid the influence of changes in the BP. In this study, we demonstrated that CAVI is not influenced by an increase in BP during measurement. In Bland-Altman plots, the mean difference of 2 measurements should be 0 if there is perfect agreement. The reproducibility of measurement of CAVI was considered to be high, because the mean difference was 0.2 and the 95% limits of agreement was within the normal tolerance. Therefore, we suggest that CAVI may be useful for routine examination and large clinical trials. Although CAVI was not affected by a change of BP during measurement, as shown in this study, hypertension is an atherosclerotic risk factors, so CAVI may be influenced by the effect of hypertension on arterial stiffness.

Study Limitation

CAVI cannot be measured accurately in patients with aortic stenosis, peripheral arterial disease, or atrial fibrillation. An ankle–brachial pressure index (ABI) <0.95 has been reported to be the cut-off value for diminished baPWV accuracy;[20] and CAVI cannot be measured accurately if the ABI is less than 0.95. Further studies are needed to evaluate the clinical value of CAVI.

In summary, baPWV and CAVI increased with aging in a general population. CAVI demonstrated a weaker correlation with SBP compared with baPWV and was not affected by an increase in BP during measurement. We conclude that CAVI may be a new and useful index of arterial distensibility and is independent of BP changes.

References

1. Asmar R, Benetos A, Topouchian J, Laurent P, Pannier B, Brisac AM, et al. Assessment of arterial distensibility by automatic pulse wave velocity measurement: Validation and clinical application studies. *Hypertension* 1995; **26:** 485–490.
2. Boutouyrie P, Tropeano AI, Asmar R, Gautier I, Benetos A, Lacolley P, et al. Aortic stiffness is an independent predictor of primary coronary events in hypertensive patients: A longitudinal study. *Hypertension* 2002; **39:** 10–15.
3. Blacher J, Asmar R, Djane S, London GM, Safar ME. Aortic pulse wave velocity as a marker of cardiovascular risk in hypertensive patients. *Hypertension* 1999; **33:** 1111–1117.
4. Laurent S, Boutouyrie P, Asmar R, Gautier I, Laloux B, Guize L, et al. Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. *Hypertension* 2001; **37:** 1236–1241.
5. Shokawa T, Imazu M, Yamamoto H, Toyofuku M, Tasaki N, Okimoto T, et al. Pulse wave velocity predicts cardiovascular mortality: Findings from the Hawaii-Los Angeles-Hiroshima study. *Circ J* 2005; **69:** 259–264.
6. Yamashina A, Tomiyama H, Takeda K, Tsuda H, Arai T, Hirose K, et al. Validity, reproducibility, and clinical significance of noninvasive brachial-ankle pulse wave velocity measurement. *Hypertens Res* 2002; **25:** 359–364.
7. Imanishi R, Seto S, Toda G, Yoshida M, Ohtsuru A, Koide Y, et al. High brachial-ankle pulse wave velocity is an independent predictor of the presence of coronary artery disease in men. *Hypertens Res* 2004; **27:** 71–78.
8. Tomiyama H, Koji Y, Yambe M, Shiina K, Motobe K, Yamada J, et al. Brachial ankle pulse wave velocity is a simple and independent predictor of prognosis in patients with acute coronary syndrome. *Circ J* 2005; **69:** 815–822.
9. Nakamura U, Iwase M, Nohara S, Kanai H, Ichikawa K, Iida M. Usefulness of brachial-ankle pulse wave velocity measurement: Correlation with abdominal aortic calcification. *Hypertens Res* 2003; **26:** 163–167.
10. Kobayashi K, Akishita M, Yu W, Hashimoto M, Ohni M, Toba K. Interrelationship between non-invasive measurements of atherosclerosis: Flow-mediated dilation of brachial artery, carotid intima-media thickness and pulse wave velocity. *Atherosclerosis* 2004; **173:** 13–18.
11. Kubo T, Miyata M, Minagoe S, Setoyama S, Maruyama I, Tei C. A simple oscillometric technique for determining new indices of arterial distensibility. *Hypertens Res* 2002; **25:** 351–358.
12. Tomiyama H, Yamashina A, Arai T, Hirose K, Koji Y, Chikamori T, et al. Influences of age and gender on results of noninvasive brachial-ankle pulse wave velocity measurement: A survey of 12517 subjects. *Atherosclerosis* 2003; **166:** 303–309.
13. Yambe T, Yoshizawa M, Saijo Y, Yamaguchi T, Shibata M, Konno S, et al. Brachio-ankle pulse wave velocity and cardio-ankle vascular index (CAVI). *Biomed Pharmacother* 2004; **58**(Suppl 1): S95–S98.
14. Shirai K, Utino J, Otsuka K, Takata M. A novel blood pressure-independent arterial wall stiffness parameter; cardio-ankle vascular index (CAVI). *J Atheroscler Thromb* 2006; **13:** 101–107.
15. Hirai T, Sasayama S, Kawasaki T, Yagi S. Stiffness of systemic arteries in patients with myocardial infarction: A noninvasive method to predict severity of coronary atherosclerosis. *Circulation* 1989; **80:** 78–86.
16. Wolinsky H. Long-term effects of hypertension on the rat aortic wall and their relation to concurrent aging changes. *Circ Res* 1972; **30:** 301–309.
17. O'Rourke MF. Arterial stiffness, systolic blood pressure, and logical treatment of arterial hypertension. *Hypertension* 1990; **15:** 339–347.
18. Hallock P. Arterial elasticity in man in relation to age as evaluated by the pulse wave velocity. *Arch Intern Med* 1934; **54:** 770–798.
19. Asmar R, Benetos A, London G, Hugue C, Weiss Y, Topouchian J, et al. Aortic distensibility in normotensive, untreated and treated hypertensive patients. *Blood Press* 1995; **4:** 48–54.
20. Motobe K, Tomiyama H, Koji Y, Yambe M, Gulinisa Z, Arai T, et al. Cut-off value of the ankle-brachial pressure index at which the accuracy of brachial-ankle pulse wave velocity measurement is diminished. *Circ J* 2005; **69:** 55–60.

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Intensive treatment of risk factors in patients with type-2 diabetes mellitus is associated with improvement of endothelial function coupled with a reduction in the levels of plasma asymmetric dimethylarginine and endogenous inhibitor of nitric oxide synthase

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Aims Vascular endothelium is a major organ involved in hyperglycaemia and is affected by plasma asymmetric dimethylarginine (ADMA). ADMA is an endogenous, competitive inhibitor of nitric oxide synthase and is induced by inflammatory cytokines of tumour necrosis factor (TNF)-α *in vitro*. We hypothesized that a tight glycaemic control may restore endothelial function in patients with type-2 diabetes mellitus (DM), in association with modulation of TNF-α and/or reduction of ADMA level.

Methods and results In 24 patients with type-2 DM, the flow-mediated, endothelium-dependent dilation (FMD: %) of brachial arteries during reactive hyperaemia was determined by a high-resolution ultrasound method. Blood samples for glucose, cholesterol, TNF-α, and ADMA analyses were also collected from these patients after fasting. No significant glycaemic or FMD changes were observed in 10 patients receiving the conventional therapy. In 14 patients who were hospitalized and intensively treated, there was a significant decrease in glucose level after the treatment [from 190 ± 55 to 117 ± 21 (mean ± SD) mg/dL, $P < 0.01$]. After the intensive control of glucose level, FMD increased significantly (from 2.5 ± 0.9 to 7.2 ± 3.0%), accompanied by a significant ($P < 0.01$) decrease in TNF-α (from 29 ± 16 to 11 ± 9 pg/dL) and ADMA (from 4.8 ± 1.5 to 3.5 ± 1.1 μM/L) levels. The changes in FMD after treatment correlated inversely with those in TNF-α ($R = -0.711$, $P < 0.01$) and ADMA ($R = -0.717$, $P < 0.01$) levels.

Conclusion The intensive correction of hyperglycaemia is associated with the improvement of endothelial function, which is coupled with the decrease in the levels of reduction of plasma TNF-α and ADMA in patients with type-2 DM. A strict glycaemic control may exert anti-cytokine and anti-atherogenic effects and may therefore be pathophysiologically important.

Introduction

Cardiovascular disease is the major cause of morbidity and mortality in patients with type-2 diabetes mellitus (DM),[1] in whom hyperglycaemia is one of the main metabolic abnormalities.[2] Blood glucose control occupies the centre stage in DM management.[3] A recent controlled trial, i.e. the United Kingdom Prospective Diabetes Study (UKPDS), suggested that an intensive glucose-lowering treatment decreases the occurrence of macrovascular complications.[4] However, the exact roles of hyperglycaemia and glycaemic control in cardiovascular complications remain to be determined in patients with type-2 DM.

Previous studies demonstrated that acute hyperglycaemia impairs endothelium-dependent vasodilation in healthy subjects[5,6] and further depresses it in patients with type-2 DM.[6] These findings indicate a possible link between glucose level and endothelial function in humans. Endothelial dysfunction is an important phenomenon in the pathogenesis of atherosclerosis[7] and is related to the derangements of nitric oxide (NO) synthase in the vessel wall.[8] Asymmetric dimethylarginine (ADMA) is an endogenous, competitive inhibitor of NO synthase.[9] Its concentration is increased by tumour necrosis

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factor-α (TNF-α),[10] which is implicated as an important factor in the pathogenesis of type-2 DM.[11]

Thus, the present study was designed to investigate whether an intensive therapy of hyperglycaemia may improve endothelial function in association with the modulation of the cytokines and/or decrease in plasma ADMA level in patients with type-2 DM.

Methods

Study patients

The study protocol was approved by the Institutional Review board, and all these patients gave their written informed consent to participate in the study. Type-2 DM was defined according to the criteria of the Diagnosis and Classification of Diabetes Mellitus.[12] Between May 1999 and June 2000, type-2 DM patients with poor glycaemic control [fasting blood glucose >200 mg/dL and/or haemoglobin A-1C (Hb A-1C) >9%] were recruited for intensive treatment of hyperglycaemia during hospitalization. Twenty-four patients were initially assessed for inclusion in the study. Among them, 14 patients [nine men and five women, mean age 61 ± 12 (SD) years] gave their consent and were admitted to the Hospital of the National Cardiovascular Center (intensive treatment group). The remaining 10 patients [seven men and three women, mean age 63 ± 15 (SD) years], who refused to be hospitalized and were obliged to keep conventional (non-intensive) diabetes treatment, served as the control group in the present study.

All the patients underwent history screening, physical examination, and laboratory analysis, including a complete blood count: the levels of plasma electrolyte, glucose, insulin, Hb A-1C, blood urea nitrogen, creatinine, transaminases and urinary protein levels, and lipid profile. Moreover, the patients were assessed for the presence of diabetic complication, i.e. retinopathy, neuropathy, nephropathy, a history of myocardial infarction, and the presence of angina pectoris and arteriosclerosis obliterans. Patients with nephrotic-range proteinuria, thyroid disease, apparent infections, or haematologic, hepatic, or renal disease were excluded from the study. Before admission, five patients had been receiving angiotensin-converting enzyme inhibitors for hypertension and five patients receiving statin for hyperlipidaemia for over 6 months. These medications were not changed throughout the study period. In addition, no new drugs other than insulin or oral hypoglycaemic agents were administered to any of these patients.

Study design

On admission, following an overnight fasting, a non-invasive assessment of brachial arterial vasoreactivity in response to reactive hyperaemia or nitroglycerin was performed with blood sampling for the determination of the levels of glucose, insulin, Hb A-1C, total cholesterol, triglyceride, HDL cholesterol, LDL cholesterol, TNF-α, and ADMA in the plasma. We also measured plasma hepatocyte growth factor (HGF) level. The HGF may protect against endothelial dysfunction, and its production is suppressed by high glucose levels.[13] Body mass index (BMI) was calculated using the formula BMI = weight (kg)/height^2 (m^2). All measurements were repeated after ~1 month of intensive treatment for hyperglycaemia.

The intensive therapy was aimed at maintaining normal fasting glucose (80–115 mg/dL) and pre-prandial blood glucose (<130 mg/dL) levels. Throughout the study, the patients followed a 1200–1300 Kcal diet regimen of 60–65 g of protein, 30–35 g of fat, and 160–170 g of carbohydrates. The level of dietary cholesterol was 350 g/day. The dose of oral anti-diabetic drugs was adjusted accordingly and/or insulin therapy was administered to improve glycaemic control. The patients were examined once or twice a week over a 4–5-week period of blood glucose monitoring. None of the patients experienced a hypoglycaemic reaction during the study.

Brachial artery ultrasound

Flow-mediated, endothelium-dependent vasodilation (FMD) following reactive hyperaemia and endothelium-independent nitroglycerin-induced vasodilation of the brachial artery were assessed using a high-resolution ultrasound machine (System Five, General Electronics) equipped with a 7.5 MHz linear array transducer.[6] After a 10 min rest in a temperature-controlled room (22–23°C), the diameter of the right brachial artery and baseline forearm flow velocity were measured. Increased forearm blood flow was induced by the inflation of a blood pressure cuff placed around the forearm to 200 mmHg or to a pressure of 50 mmHg greater than the systolic blood pressure. This was followed by deflation (RD2 Cuff Deflator, Hokanson Inc., Bellevue, WA, USA) after 5 min. Repeated blood flow scans were obtained to measure the diameter of the brachial artery. After 15 min of vessel recuperation, a repeated measurement of the diameter of the resting brachial artery and repeated blood flow scans were obtained. Sublingual nitroglycerin (0.4 mg) was administered, and then final scans were obtained after 3 min. Throughout the study, a single lead electrocardiogram was obtained, and blood pressure was measured in the left arm every 2 min by an automated blood pressure recorder.

Ultrasound images were recorded on an S-VHS videocassette recorder. Depth and gain settings were used to optimize the images of the lumen–arterial wall interface. Vessel diameter was measured in triplicate at end diastole, from the anterior to the posterior interface between the media and the adventitia. Flow-mediated vasodilation was calculated as the ratio of brachial artery diameter after reactive hyperaemia to baseline diameter and expressed as a per cent increase. Nitroglycerin-mediated vasodilation was calculated in an analogous manner. Volumetric flow rate was calculated by multiplying the time velocity integral of the angle (~70°)-corrected Doppler flow signal by the heart rate and the vessel cross-sectional area. Changes in blood flow were expressed as the percentages of the resting flow measurements. All measurements were performed with the observers blind to patient information and study date. Using this methodology and analysis, the intra- and inter-observer variabilities in brachial artery diameter were 0.03 ± 0.02 (mean ± SD) and 0.06 ± 0.02 mm, respectively, and the variability in FMD performed on two different days was 1.4 ± 0.5%.

Laboratory measurements

Fasting plasma glucose level was measured by the glucose oxidase method and Hb A-1C level was measured by automated high-performance liquid chromatography. Insulin level was measured by the conventional radioimmunoassay. To assess insulin resistance, we used the following homeostasis model assessment (HOMA) parameters: HOMA-R = [fasting blood glucose (mg/dL) × fasting insulin (μU/mL)]/405.[14]

Total cholesterol, triglyceride, and HDL cholesterol levels were determined as described previously.[15] LDL cholesterol level was calculated using the Friedewald equation.[16]

TNF-α and HGF levels were determined by enzyme-linked immunosorbent assay (Otsuka Pharmaceutical Co., Tokushima, Japan). The detection limits of these methods are 2 pg/mL for TNF-α and 0.1 ng/mL for HGF. The intra- and inter-assay coefficients of variation were both ~7% for the enzyme-linked immunosorbent assay.

Plasma ADMA concentration was measured using high-performance liquid chromatography with pre-column derivatization, as previously described.[17] In brief, equilibrated CBA columns

Table 1 Patient characteristics

	Standard therapy (control), $n = 10$	Intensive therapy, $n = 14$	*P*-value
Age (years), mean ± SD	63 ± 15	61 ± 12	0.7201
Male, *n* (%)	7 (70%)	9 (64%)	0.7697
Risk factors			
Hypertension, *n* (%)	5 (50%)	7 (50%)	1.000
Hyperlipidaemia, *n* (%)	6 (60%)	7 (50%)	0.9448
Smoking, *n* (%)	4 (40%)	5 (36%)	0.8307
Retinopathy, *n* (%)	2 (20%)	2 (14%)	0.7111
Proteinuria, *n* (%)	2 (20%)	4 (29%)	0.6326
CAD, *n* (%)	2 (20%)	3 (21%)	0.9323
Peripheral artery disease, *n* (%)	0 (0%)	2 (14%)	0.6175
Stroke, *n* (%)	1 (10%)	1 (7%)	0.8028
Medications, baseline[a]			
ACE-inhibitor, *n* (%)	4 (40%)	5 (36%)	0.8307
Calcium blocker, *n* (%)	2 (20%)	1 (7%)	0.7543
Beta-blocker, *n* (%)	2 (20%)	1 (7%)	0.7543
Statin, *n* (%)	6 (60%)	5 (36%)	0.4462
Sulphonylurea, *n* (%)	7 (70%)	10 (71%)	0.9395
Biguanide, *n* (%)	1 (10%)	1 (7%)	0.7543
α-Glucosidase inhibitor, *n* (%)	4 (40%)	2 (14%)	0.3380

ACE, angiotensin-converting enzyme.
[a]Medications immediately before additional therapy for dysglycaemia.

(Bond Elut, Varian Inc., CA, USA) were used for three-fold washing with 1 mL serum samples with methanol and distilled water. Thereafter, the samples were eluted with 10% ammonia and dried. The sediment obtained was dissolved in 1 mL of water, the solution was centrifuged, and the supernatant was subjected to high-performance liquid chromatography using ODS columns (Fisher Scientific, St Louis, MO, USA). ADMA concentration was calculated on the basis of the recovery rate of L-monomethyl-arginine (Sigma, St Louis, MO, USA), used as the internal standard. Intra- and inter-assay variabilities were both ~6%, with a detection limit of 0.1 μM/L.

Statistical analyses

Sample size calculations were performed using a primary endpoint variable of FMD. Power calculations indicated that to detect a mean difference in FMD of 4% (SD, 3%), 13 subjects would be needed to complete the study (α statistics, 0.05; power >0.9). All data are expressed as mean ± SD. Two-tailed *t*-tests or the Mann-Whitney *U* test was used to compare the changes in response to treatment. To compare the proportions of patients, Fisher's exact test was used. Linear regression curves and correlations were calculated according to the least-squares method. *P*-values less than 0.05 were considered significant.

Results

The baseline characteristics of 10 control patients who received standard therapy and 14 intensively treated patients are summarized in *Table 1*. All 24 patients completed 3–4-week follow-up measurements.

The control patients were treated by diet alone (three patients) or diet plus oral hypoglycaemic agents (an increased dose of sulphonylurea, six patients and addition of metformin to sulphonylurea, one patient). *Table 2* shows no significant improvements in clinical and biochemical parameters during the observation period of 28 ± 5 days of standard therapy. Neither the fasting blood glucose (from 181 ± 42 to 186 ± 38 mg/dL) nor the response of FMD to reactive hyperaemia (from 3.0 ± 1.3 to 2.6 ± 1.0%) changed.

Table 2 Changes in biochemical and clinical parameters before and after standard treatment of hyperglycaemia in 10 control patients with type-2 DM

	Before	After	*P*-value
Hb A-1C (%)	9.4 ± 2.2	9.4 ± 2.0	>0.999
Insulin (μU/mL)	4.2 ± 2.0	4.4 ± 2.2	0.834
HOMA-R	1.9 ± 1.2	1.8 ± 1.0	0.842
Total cholesterol (mg/dL)	212 ± 28	210 ± 25	0.868
TG (mg/dL)	128 ± 40	129 ± 45	0.959
HDL cholesterol (mg/dL)	50 ± 19	51 ± 20	0.910
LDL cholesterol (mg/dL)	128 ± 22	127 ± 25	0.925
Systolic BP (mmHg)	139 ± 18	138 ± 20	0.908
Diastolic BP (mmHg)	76 ± 8	78 ± 10	0.627
BMI (kg/m^2)	23.8 ± 2.7	23.4 ± 3.1	0.763

TG, triglyceride; BP, blood pressure. Values are expressed as mean ± SD.

Biochemical and clinical changes after intensive treatment of hyperglycaemia

In the intensive therapy group, the patients were all treated by diet alone (three patients), diet plus oral hypoglycaemic agents (sulphonylurea newly given, one patient; an increased dose of sulphonylurea, one patient; addition of metformin to sulphonylurea, two patients; and addition of α-glucosidase inhibitor to sulphonylurea, one patient), or diet plus insulin (switched from oral hypoglycaemic agents, six patients). The duration of intensive treatment of hyperglycaemia was 34 ± 13 days. Clinical and biochemical parameters at baseline (before treatment) were similar between the standard therapy group and the intensive therapy group (*Tables 2* and *3*). After the intensive

Table 3 Changes in biochemical and clinical parameters before and after intensive treatment of hyperglycaemia in 14 patients with type-2 DM

	Before	After	*P*-value	*P*-value (vs. control after)
Hb A-1C (%)	9.7 ± 1.6	8.6 ± 1.4	0.032	0.287
Insulin (μU/mL)	4.4 ± 2.6	5.3 ± 2.0	0.314	0.233
HOMA-R	2.0 ± 1.1	1.6 ± 0.5	0.226	0.524
Total cholesterol (mg/dL)	202 ± 33	173 ± 28	0.019	0.003
TG (mg/dL)	121 ± 43	105 ± 51	0.378	0.246
HDL cholesterol (mg/dL)	52 ± 21	52 ± 17	>0.999	0.896
LDL cholesterol (mg/dL)	125 ± 25	101 ± 29	0.027	0.032
Systolic BP (mmHg)	134 ± 18	128 ± 14	0.779	0.1626
Diastolic BP (mmHg)	77 ± 7	74 ± 8	0.301	0.2880
BMI (kg/m^2)	23.6 ± 3.6	21.4 ± 3.2	0.049	0.1405

Values are expressed as mean ± SD.

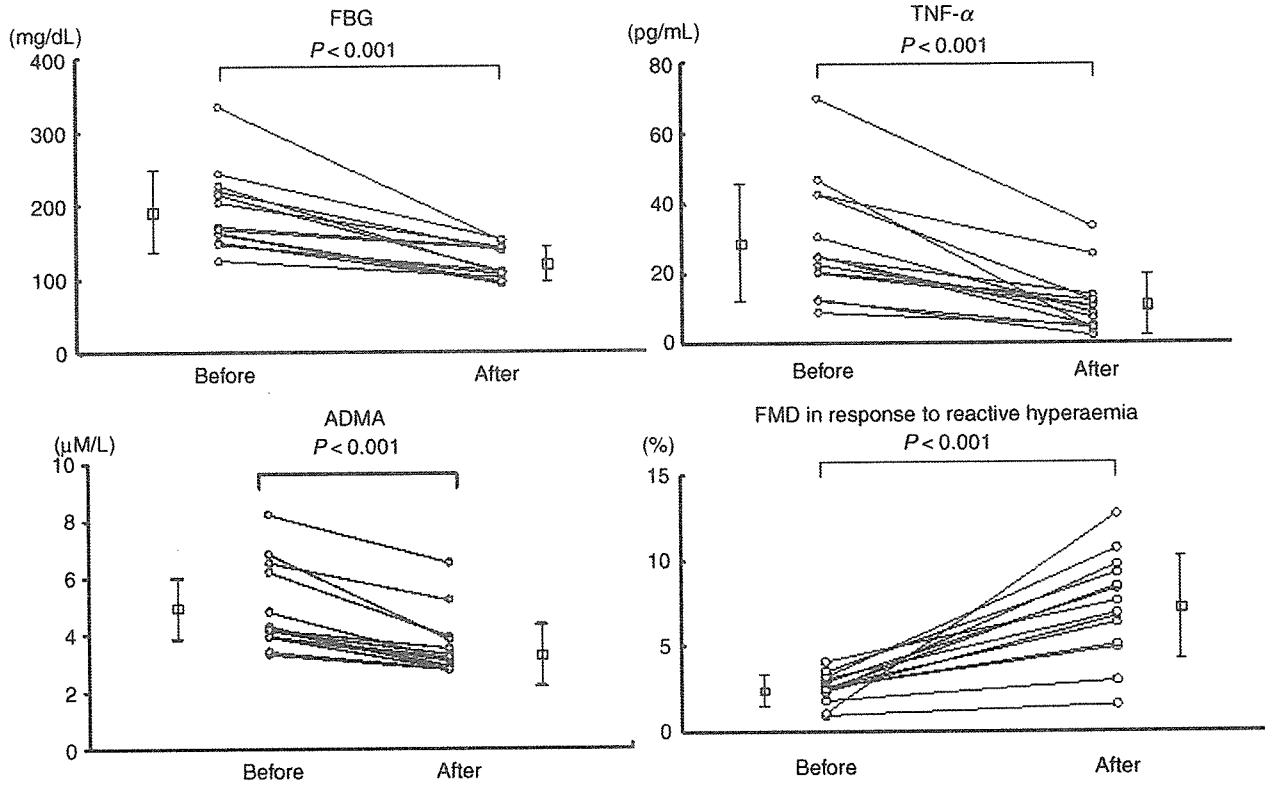

Figure 1 Individual measurements of fasting blood glucose (FBG), TNF-α and ADMA levels, and FMD in response to reactive hyperaemia before and after intensive treatment of hyperglycaemia in 14 patients with type-2 DM.

treatment, the fasting glucose level significantly decreased from 190 ± 55 to 117 ± 21 mg/dL ($P < 0.001$), as shown in *Figure 1*. Significant decreases in Hb A-1C, total cholesterol, and LDL cholesterol levels and BMI were observed, whereas no changes in HOMA-R index; insulin, triglyceride, or HDL cholesterol levels; and systolic and diastolic blood pressures were observed (*Table 3*). Two of three patients with coronary artery disease were taking statins at the time of the study.

The levels of plasma TNF-α (from 29 ± 16 to 11 ± 9 pg/dL, $P < 0.001$) and ADMA (from 4.8 ± 1.5 to 3.5 ± 1.1 μM/L, $P < 0.001$) significantly decreased after the intensive control of glucose level (*Figure 1*). However, HGF level did not significantly change throughout the study (from 0.19 ± 0.05 to 0.20 ± 0.08 ng/mL).

Brachial artery reactivity after intensive treatment of hyperglycaemia

Before treatment under hyperglycaemic condition, the baseline brachial arterial diameter was 4.5 ± 0.3 mm, and FMD in response to reactive hyperaemia was 2.4 ± 0.9%. After the intensive control of glucose level, FMD significantly ($P < 0.001$) increased to 7.2 ± 3.1% (*Figure 1*), whereas the baseline diameter (4.5 ± 0.2 mm) did not change. There was a similar increase in blood flow during reactive

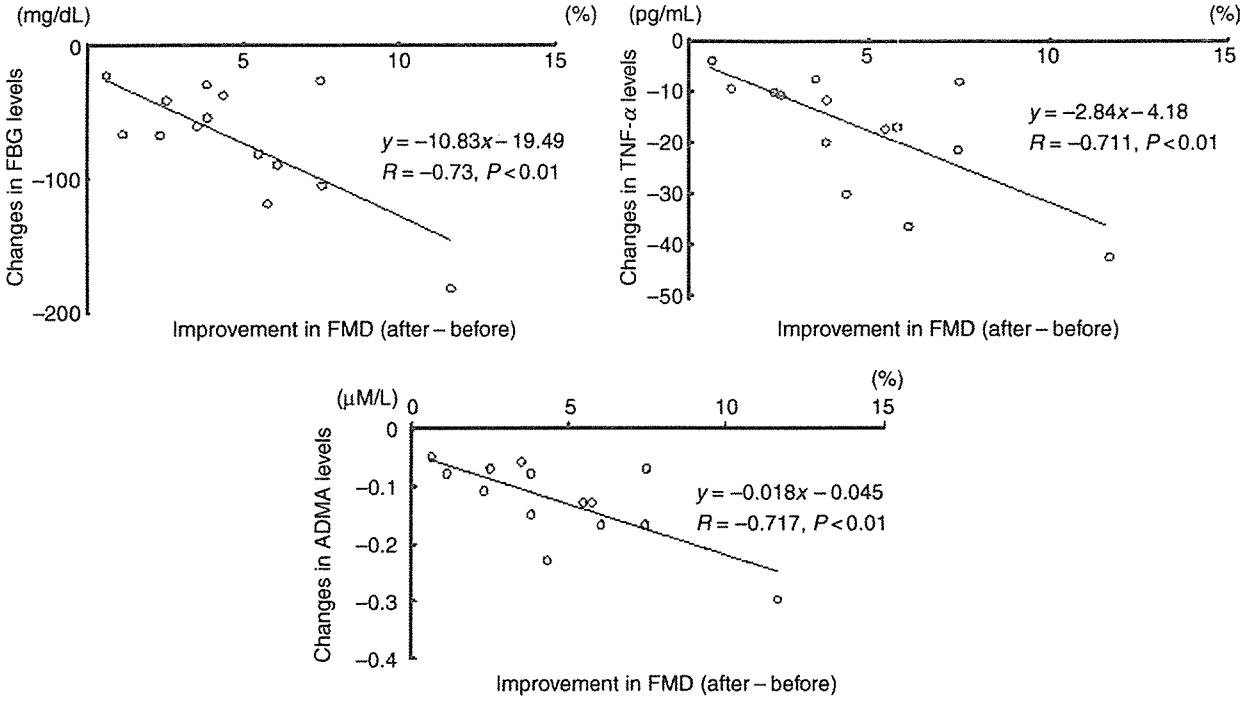

Figure 2 Correlation of improvement of FMD after treatment with decreases in levels of FBG, TNF-α, and ADMA.

hyperaemia (293 ± 16 vs. 296 ± 20%) and a similar baseline heart rate (67 ± 7 vs. 65 ± 8 bpm) before and after the treatment.

Nitroglycerin-mediated vasodilation was 9.8 ± 1.0% before treatment; however, in contrast to FMD, it did not change after treatment (10.0 ± 1.6%).

Correlation with FMD improvement

As shown in *Figure 2*, the improvement of FMD after treatment correlated inversely with the changes in fasting glucose ($R = -0.730$, $P < 0.01$), TNF-α ($R = -0.711$, $P < 0.01$), and ADMA ($R = -0.717$, $P < 0.01$) levels. However, the improvement of FMD did not correlate significantly with the changes in Hb A-1C level ($R = 0.408$, $P = 0.148$), total cholesterol level ($R = 0.325$, $P = 0.256$), or BMI ($R = 0.270$, $P = 0.351$).

Six-to-12-month follow-up

A follow-up study was performed 6–12 months after the discharge. In eight of 14 patients with an Hb A-1C level of <8.0% at this follow-up period, fasting blood glucose level and FMD remained at 127 ± 26 mg/dL and 8.4 ± 1.0%, respectively. In contrast, in the remaining six patients with an Hb A-1C level of ≥8.0%, fasting blood glucose level and FMD worsened to be 178 ± 30 mg/dL and 3.1 ± 1.1%, respectively. There were inverse correlations of FMD with fasting blood glucose ($R = -0.577$) and Hb A-1C levels ($R = -0.860$).

Discussion

The major finding in the present study is that the intensive treatment of hyperglycaemia is associated with the improvement of endothelial function, coupled with the decrease in plasma TNF-α and ADMA (an endogenous inhibitor of NO synthase) levels in patients with type-2 DM.

Previous studies revealed that an acute increase in blood glucose level impairs endothelium-dependent vasodilation in healthy subjects[5,6] and further inhibits it in patients with type-2 DM.[6] DM is a state of chronic hyperglycaemia, and glycaemic control is one of the major goals of diabetes management.[18] As shown in *Figure 1*, endothelial dysfunction improves after a 5-week intervention targeting hyperglycaemia in type-2 diabetes patients, accompanied by a relatively small but significant decrease in Hb A-1C level. In contrast, either hyperglycaemia or endothelial function did not change in control outpatients who received routine treatment. These findings suggest that hyperglycaemia may be a fundamental abnormality underlying the mechanism that causes endothelial dysfunction in DM. However, we must acknowledge a potential limitation that an appropriate control group should have included patients who were admitted to the hospital, but did not receive intensive treatment. In addition, the number of statistical tests performed and relatively small sample size of the study population may potentially infiltrate type-I error.

In patients with type-2 DM, TNF-α levels were elevated in both blood and tissue.[19–21] Taken together with results from knockout mice deficient in TNF-α or its receptors,[11] it is suggested that TNF-α is a factor contributing to the pathogenesis of type-2 DM. Hyperglycaemia is an important stimulus for TNF-α synthesis in human peripheral monocytes *in vitro*.[22] A previous *in vivo* study demonstrated that the administration of TNF-α impairs endothelial-dependent vasodilation in rats.[23] In the present study, as shown in *Figure 1*, plasma TNF-α level decreased after the intensive treatment of hyperglycaemia. This finding indicates the therapeutic potential of a strict glycaemic control against inflammatory cytokines that play a prominent role in atherogenesis.[7]

TNF-α and hyperglycaemia could impair dimethylarginine dimethylaminohydrolase and cause the accumulation of ADMA, an endogenous, competitive inhibitor of NO synthase, contributing to the derangements of NO pathways in the vessel.[10,24] The intra-arterial infusions of ADMA significantly impair endothelium-dependent flow responses in the human forearm.[25] In the present study, we found that the ADMA level increased in patients with type-2 DM (*Figure 1*), and its decrease after the strict glycaemic control correlated significantly with the improvement of FMD (*Figure 2*). Not only ADMA, but also TNF-α itself downregulates NO synthase by decreasing mRNA's half-life.[26] Moreover, both inflammatory cytokines and high glucose levels enhance the production of oxygen-derived free radicals,[27,28] which rapidly inactivate NO.[29] In patients with type-2 DM, the extent of urinary excretion of the isoprostanes (8-iso-prostaglandin $F_{2\alpha}$) significantly decreased ~4 weeks after an intensive therapy for hyperglycaemia, an intervention similar to that used in the present study.[30] Taking together a recent report that lowering serum TNF-α level alone (without glycaemic control) does not improve endothelial function,[31] these findings suggest that the hyperglycaemia-induced oxidative stress could be a key factor in the pathophysiology of diabetes.

HGF is characterized to be one of the most potent mitogens among the growth factors for vascular endothelial cells and contributes to vascular protection or repair.[13] Because its production is suppressed by glucose in a dose-dependent manner *in vitro*,[13] we hypothesized that endothelial dysfunction might be associated with the decreased production of HGF in diabetic patients. However, this was not the case. The level of HGF did not change throughout this study. Moreover, as shown in *Table 3*, insulin sensitivity, as assessed using HOMA-R index,[14] did not change significantly. Insulin resistance contributes, in part, to the pathogenesis of type-2 DM and may be potentially linked with endothelial dysfunction and ADMA.[32] To address this important issue, we need to further assess insulin sensitivity with a more specific method such as steady-state plasma glucose measurement.

Impaired endothelium is a key factor for diabetic macroangiopathy.[7] Thus, restoring endothelial function has important clinical implications for reducing the risk of cardiovascular diseases in diabetic patients. The present results, although obtained in a short period, suggest that a long-term maintenance of strict glycaemic control is important. If hyperglycaemia continues, then the expression level of NO synthase and the generation of NO may be chronically reduced, leading to a persistent dysfunction of the vascular endothelium and the consequent atherogenesis. In the UKPDS conducted for more than 15 years,[4] the difference in Hb A-1C level between the conventionally and intensively treated groups was significant throughout the study. However, Hb A-1C level progressively increased in both groups. The median Hb A-1C level was 6.6% in the first 5 years, but increased to 8.1% in the last 5 years, even in the intensively treated group. A difficulty in maintaining a good glycaemic control may explain, in part, the borderline decrease in the extent of myocardial infarction ($P = 0.05$) induced by the intensive treatment. Taking the multifactorial aetiology of macrovascular disease into account, the results of the UKPDS also suggest that the optimum treatment of patients with type-2 DM would include the control of blood pressure and correction of lipid abnormalities in addition to the control of glucose level. For the assessment of the effectiveness of therapeutic/dietary interventions and for the early detection of vascular dysfunction, plasma ADMA may be useful as a potential biochemical marker.[9,33] Metformin,[34] angiotensin-converting enzyme inhibitors/angiotensin II receptor blocker,[35] and statins[36] could decrease ADMA level. Although these drugs were not newly given in the present patients, it is possible that an increased utilization of and compliance with medications and an improved diet during hospitalization may contribute, at least in part, to endothelial function improvement. Insulin-sensitizing rosiglitazone also decreases ADMA level.[37] A recent study has suggested that obese and insulin resistance are not strongly associated with the development of type-2 DM in Japanese patients with a BMI of ~23 kg/m^2 (from the Japan Diabetes Complications Study), unlike in European patients with a BMI of ~29 kg/m^2 (from the UKPDS).[38]

In conclusion, in patients with type-2 DM, the intensive treatment of hyperglycaemia is associated with the improvement of endothelial dysfunction, coupled with decreases in TNF-α and ADMA levels. A strict glycaemic control may exert anti-cytokine and anti-atherogenic effects and may therefore be pathophysiologically important.

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Conflict of interest: none declared.

References

1. Haffner SM, Lehto S, Ronnemaa T, Pyorala K, Laakso M. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med* 1998;339:229–234.
2. 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;355:773–778.
3. Grundy SM, Benjamin IJ, Burke GL, Chait A, Eckel RH, Howard BV, Mitch W, Smith SC Jr, Sowers JR. Diabetes and cardiovascular disease: a statement for healthcare professionals from the American Heart Association. *Circulation* 1999;100:1134–1146.
4. UK Prospective Diabetes Study (UKPDS) Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 1998;352:837–853.
5. Williams SB, Goldfine AB, Timimi FK, Ting HH, Roddy MA, Simonson DC, Creager MA. Acute hyperglycemia attenuates endothelium-dependent vasodilation in humans *in vivo*. *Circulation* 1998;97:1695–1701.
6. Kawano H, Motoyama T, Hirashima O, Hirai N, Miyao Y, Sakamoto T, Kugiyama K, Ogawa H, Yasue H. Hyperglycemia rapidly suppresses flow-mediated endothelium-dependent vasodilation of brachial artery. *J Am Coll Cardiol* 1999;34:146–154.
7. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999; 340:115–126.
8. Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. *J Clin Invest* 1997;100:2153–2157.
9. Cooke JP. Does ADMA cause endothelial dysfunction? *Arterioscler Thromb Vasc Biol* 2000;20:2032–2037.

10. Ito A, Tsao PS, Adimoolam S, Kimoto M, Ogawa T, Cooke JP. Novel mechanism for endothelial dysfunction: dysregulation of dimethylarginine dimethylaminohydrolase. *Circulation* 1999;**99**:3092-3095.
11. Moller DE. Potential role of TNF-alpha in the pathogenesis of insulin resistance and type 2 diabetes. *Trends Endocrinol Metab* 2000;**11**: 212-217.
12. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 1997;**20**:1183-1197.
13. Morishita R, Nakamura S, Nakamura Y, Aoki M, Moriguchi A, Kida I, Yo Y, Matsumoto K, Nakamura T, Higaki J, Ogihara T. Potential role of an endothelium-specific growth factor, hepatocyte growth factor, on endothelial damage in diabetes. *Diabetes* 1997;**46**:138-142.
14. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985;**28**:412-419.
15. Shinozaki K, Suzuki M, Ikebuchi M, Takaki H, Hara Y, Tsushima M, Harano Y. Insulin resistance associated with compensatory hyperinsulinemia as an independent risk factor for vasospastic angina. *Circulation* 1995;**92**:1749-1757.
16. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972;**18**:499-502.
17. Pettersson A, Uggla L, Backman V. Determination of dimethylated arginines in human plasma by high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* 1997;**692**:257-262.
18. Keen H, Clark C, Laakso M. Reducing the burden of diabetes: managing cardiovascular disease. *Diabetes Metab Res Rev* 1999;**15**:186-196.
19. Winkler G, Salamon F, Harmos G, Salamon D, Speer G, Szekeres O, Hajos P, Kovacs M, Simon K, Cseh K. Elevated serum tumor necrosis factor-alpha concentrations and bioactivity in type 2 diabetics and patients with android type obesity. *Diabetes Res Clin Pract* 1998;**42**(Suppl.):169-174.
20. Zinman B, Hanley AJ, Harris SB, Kwan J, Fantus IG. Circulating tumor necrosis factor-alpha concentrations in a native Canadian population with high rates of type 2 diabetes mellitus. *J Clin Endocrinol Metab* 1999;**84**:272-278.
21. Clausell N, Kalil P, Biolo A, Molossi S, Azevedo M. Increased expression of tumor necrosis factor-alpha in diabetic macrovasculopathy. *Cardiovasc Pathol* 1999;**8**:145-151.
22. Morohoshi M, Fujisawa K, Uchimura I, Numano F. Glucose-dependent interleukin 6 and tumor necrosis factor production by human peripheral blood monocytes *in vitro*. *Diabetes* 1996;**45**:954-959.
23. Wang P, Ba ZF, Chaudry IH. Administration of tumor necrosis factor-alpha *in vivo* depresses endothelium-dependent relaxation. *Am J Physiol* 1994;**266**:H2535-H2541.
24. Lin KY, Ito A, Asagami T, Tsao PS, Adimoolam S, Kimoto M, Tsuji H, Reaven GM, Cooke JP. Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase. *Circulation* 2002;**106**:987-992.
25. Calver A, Collier J, Leone A, Moncada S, Vallance P. Effect of local intra-arterial asymmetric dimethylarginine (ADMA) on the forearm arteriolar bed of healthy volunteers. *J Hum Hypertens* 1993;**7**:193-194.
26. Yoshizumi M, Perrella MA, Burnett JC Jr, Lee ME. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. *Circ Res* 1993;**73**:205-209.
27. Matsubara T, Ziff M. Increased superoxide anion release from human endothelial cells in response to cytokines. *J Immunol* 1986;**137**: 3295-3298.
28. Marfella R, Quagliaro L, Nappo F, Ceriello A, Giugliano D. Acute hyperglycemia induces an oxidative stress in healthy subjects. *J Clin Invest* 2001;**108**:635-636.
29. Gryglewski RJ, Palmer RM, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* 1986;**320**:454-456.
30. Davi G, Ciabattoni G, Consoli A, Mezzetti A, Falco A, Santarone S, Pennese E, Vitacolonna E, Bucciarelli T, Costantini F, Capani F, Patrono C. *In vivo* formation of 8-iso-prostaglandin f2alpha and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation. *Circulation* 1999;**99**:224-229.
31. Bilsborough W, O'Driscoll G, Stanton K, Weerasooriya R, Dembo L, Taylor R, Green D. Effect of lowering tumour necrosis factor-alpha on vascular endothelial function in type II diabetes. *Clin Sci* 2002;**103**:163-169.
32. Nash DT. Insulin resistance, ADMA levels, and cardiovascular disease. *JAMA* 2002;**287**:1451-1452.
33. Fard A, Tuck CH, Donis JA, Sciacca R, Di Tullio MR, Wu HD, Bryant TA, Chen NT, Torres-Tamayo M, Ramasamy R, Berglund L, Ginsberg HN, Homma S, Cannon PJ. Acute elevations of plasma asymmetric dimethylarginine and impaired endothelial function in response to a high-fat meal in patients with type 2 diabetes. *Arterioscler Thromb Vasc Biol* 2000;**20**:2039-2044.
34. Asagami T, Abbasi F, Stuelinger M, Lamendola C, McLaughlin T, Cooke JP, Reaven GM, Tsao PS. Metformin treatment lowers asymmetric dimethylarginine concentrations in patients with type 2 diabetes. *Metabolism* 2002;**51**:843-846.
35. Delles C, Schneider MP, John S, Gekle M, Schmieder RE. Angiotensin converting enzyme inhibition and angiotensin II AT1-receptor blockade reduce the levels of asymmetrical N(G), N(G)-dimethylarginine in human essential hypertension. *Am J Hypertens* 2002;**15**:590-593.
36. Lu TM, Ding YA, Leu HB, Yin WH, Sheu WH, Chu KM. Effect of rosuvastatin on plasma levels of asymmetric dimethylarginine in patients with hypercholesterolemia. *Am J Cardiol* 2004;**94**:157-161.
37. Stuhlinger MC, Abbasi F, Chu JW, Lamendola C, McLaughlin TL, Cooke JP, Reaven GM, Tsao PS. Relationship between insulin resistance and an endogenous nitric oxide synthase inhibitor. *JAMA* 2002;**287**:1420-1426.
38. Sone H, Ito H, Ohashi Y, Akanuma Y, Yamada N; Japan Diabetes Complication Study Group. Obesity and type 2 diabetes in Japanese patients. *Lancet* 2003;**361**:85.

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Signaling pathway of nitric oxide production induced by ginsenoside Rb1 in human aortic endothelial cells: A possible involvement of androgen receptor

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Abstract

Ginsenosides have been shown to stimulate nitric oxide (NO) production in aortic endothelial cells. However, the signaling pathways involved have not been well studied in human aortic endothelial cells. The present study was designed to examine whether purified ginsenoside Rb1, a major active component of ginseng could actually induce NO production and to clarify the signaling pathway in human aortic endothelial cells. NO production was rapidly increased by Rb1. The rapid increase in NO production was abrogated by treatment with nitric oxide synthetase inhibitor, L-NAME. Rb1 stimulated rapid phosphorylation of Akt (Ser473), ERK1/2 (Thr202/Thr204) and eNOS (Ser1177). Rapid phosphorylation of eNOS (Ser1177) was prevented by SH-5, an Akt inhibitor or wortmannin, PI3-kinase inhibitor and partially attenuated by PD98059, an upstream inhibitor for ERK1/2. Interestingly, NO production and eNOS phosphorylation at Ser1177 by Rb1 were abolished by androgen receptor antagonist, nilutamide. The results suggest that PI3kinase/Akt and MEK/ERK pathways and androgen receptor are involved in the regulation of acute eNOS activation by Rb1 in human aortic endothelial cells.

Keywords: Ginsenoside Rb1; Endothelial cells; Nitric oxide; eNOS; Androgen receptor; P13-kinase; Akt; ERK; MEK; Phosphorylation

Ginseng, the root of Panax ginseng C.A. Meyer (Araliaceae), is a well-known and popular herbal medicine used worldwide. Among more than 30 ginsenosides, the active ingredient of ginseng, ginsenoside Rb1 is regarded as the main compound responsible for many pharmaceutical actions of ginseng. The oral administration of ginseng caused a decrease in blood pressure in essential hypertension [1]. Intravenous administration of ginsenosides (a mixture of saponin from Panax ginseng C.A. Meyer) lowered blood pressure in a dose-dependent manner in anesthetized rats [2]. Although these reports suggest that ginsenosides could stimulate the production of nitric oxide (NO) by aortic vascular endothelial cells, the precise mechanisms of the ginsenoside actions have not been fully elucidated [3]. NO released from endothelial cells via the endothelial nitric oxide synthetase (eNOS) is a pivotal vasoprotective molecule. In addition to its vasodilating feature, endothelial NO has anti-atherosclerotic properties, such as inhibition of platelet aggregation, leukocyte adhesion, smooth muscle cell proliferation, and expression of genes involved in atherosclerosis [4].

The present study aims at investigating the signaling pathways involved in NO production by purified ginsenoside Rb1 in human aortic endothelial cells in vitro.

Materials and methods

Materials. Rb1, nilutamide, L-NAME (hydrochloride), Hanks' balanced salt solution (HBSS) were purchased from Sigma (St. Louis, MO,

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USA). ICI182780 was from Zeneca Pharmaceuticals (Macdesfield, UK). 4,5-diaminofluorescin diacetate (DAF-2 DA) was purchased from Daiichi (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan). PD98059, SH-5, wortmannin and Nitric Oxide Synthase Assay Kit were from Calbiochem (EDM Biosciences, Inc., La Jolla, CA, USA and Germany). L-[^{3}H]Arginine was purchased from Amersham (Amersham Biosciences, Uppsala, Sweden). Antibody of phospho-eNOS (Ser1177) was from upstate (Upstate Inc., Lake Placid, NY). Antibody for eNOS/NOS type III was purchased form BD Transduction Laboratories (BD Biosciences, Franklin Lakes, NJ, USA). All other antibodies were form Cell Signaling Technology (Beverly, MA, USA). LumiGLO Reserve Chemiluminescent Substrate Kit was from KPL (Gaithersburg, MD, USA). EBM-2 (endothelial cell base medium) was from Clonetics (Walkersville, MD, USA). Human aortic endothelial cells (HAECs) were from Cambrex (Cambrex BioScience Walkersville, Inc. Walkersville, MD, USA). Fetal bovine serum (FBS) was from CCT (Sanko Junyaku Co., Ltd, Tokyo, Japan), Fetal bovine serum charcoal stripped was from MultiSer (ThermoTrace Ltd., Melbourne, Australia).

Cell culture. HAECs were cultured in a 37 °C humidified atmosphere of 95% air/5% CO_2 in EGM-2 (endothelial cell grows medium 2) medium supplemented with 10% FBS. The EGM-2 medium consisted of 0.1% EGF, 0.04% hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R^3-IGF-1, 0.1% ascorbic acid, 0.1% GA-1000, and 0.1% heparin. Experiments were performed with cells from passages 5 to 7. For all experiments, HAECs were plated at a concentration of 1×10^4/mL and grown to confluence. Then cells were serum-starved for 6 h in phenol red free EBM-2 containing 1% DCC-FBS, that was removed the steroid by processing it with dextran-coated charcoal (DCC-FBS). In some inhibitory experiments, the inhibitors were added to cells 60 min before the stimuli. DMSO was used as a solvent for Rb1, PD98059, wortmannin, SH-5, L-NAME, nilutamide, and DAF-2 DA present at equal concentrations (0.01%) in all groups, including the vehicle.

Western blot analysis. After treatment with reagents, confluent monolayers of cells were washed two times in ice-cold phosphate-buffered saline and lysed with buffer containing 20 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton-X, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/LNa_3VO_4, 1 μg/mL Leupeptin, 1 mM PMSF). For western blot analysis, total cell lysate was subjected to SDS–polyacrylamide gel electrophoresis (PAGE), and proteins were transferred to poly vinilidene difluoride (PVDF) membrane. The antibodies used in this study were anti-phospho-ERK1/2 (Thr202/Thr204), anti-ERK1/2, anti phospho-Akt (Ser473), anti-Akt, anti-phosph-eNOS (ser1177) and anti-NOS. Antibodies were detected by means of a horseradish peroxidase-linked secondary antibody and immunoreactive bands were visualized using LumiGLO Reserve Chemiluminescent Substrate Kit.

Endothelial NO synthase activity assay. Endothelial cell NO synthase (eNOS) activity was quantified by measuring the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline by the use of a NO synthase assay kit.

Measurement of intracellular production of NO. Production of NO was assessed using the NO-sensitive fluorescent dye DAF-2 DA [5]. Briefly, confluent cells were serum-staved for 6 h. Because NOS generates O_2^- instead of NO in the absence of L-arginine, so L-arginine (100 μmol/L) was add 1 h prior to all solutions, except for the experiment with *N*-nitro-L-arginine methyl ester (L-NAME; a NOS inhibitor)-treated cells. Cells were loaded with DAF-2 DA (final concentration 5 μmol/L, 30 min 37 °C) and than rinsed three times with HBSS, kept in the dark, and maintained at 37 °C in 1% EBM-2 medium with a warming stage. After 30 min, cells were then treated with Rb1 or other stimuli. In some inhibitory experiments, the inhibitors were administered 30 min before loading with DAF-2 DA. Green fluorescence intensity was measured with a laser scanning confocal microscopy system (LSCM) (Bio-Rad Laser Sharp2000). The fluorescence image was obtained as a 512×512 pixel frame. Ex = 488 nm, EM = 510 nm. All other settings, including scanning speed, pinhole diameter, and voltage gain, remained the same for all experiments.

Statistics. Data are means ± SEM. Statistical comparisons were performed by Student's *T* test between two groups. A value of $P < 0.05$ was considered significant.

Results

Rb1 stimulates rapid production of NO in human aortic endothelia cells

We used the NO-specific fluorescent dye DAF-2 DA to evaluate the effect of Rb1 on NO production in HAECs. 5, 10, 15, 30, 60, 120 and 180 min after Rb1 treatment, cells were fixed and then viewed using a fluorescence microscope. Emission of green light (510 nm) from cells excited by light at 488 nm is indicative of NO production. A significant increase in green fluorescence was observed >15 min after the addition of Rb1 and lasted for 60 min in HAECs (Fig. 1A). Maximal stimulation of NO production was obtained at 30 min.

To verify that the rapid increase in green fluorescence in response to Rb1 treatment specifically reflected NO production, we compared results from HAECs treated with acetylcholine (1 μmol/L) or Rb1 (1 μmol/L) for 5 min. Reassuringly, treatment with either acetylcholine and calcium ionophore or Rb1 resulted in a increase in green fluorescence (Fig. 1B). We next examined the effects of the NOS inhibitor L-NAME to determine whether the NO increase was attributable to NOS derived de novo synthesis. As shown in Fig. 1C, the Rb1-induced DAF-2 DA fluorescence was completely suppressed by pretreatment with L-NAME (0.5 mmol/L). The results suggested that the rapid increase in NO production after Rb1 treatment was mediated by an increase in NOS activity.

Rb1 stimulates phosphorylation of eNOS (Ser1177) and increases NOS activity

To examine involvement of eNOS in the NO increase, the effect of Rb1 on eNOS phosphorylation at Ser-1177 was tested by Western blotting. As shown in Fig. 2, Rb1 induced rapid eNOS phosphorylation after 10 min of incubation, maximal eNOS phosphorylation by Rb1 was observed from 30 to 60 min of incubation. The relative magnitude of eNOS phosphorylation falls subsequently but is still significantly greater than control after 120 min of Rb1 incubation (Fig. 2A, upper blots). The acute effect by Rb1 on eNOS phosphorylation was concentration dependent (Fig. 2B, upper blots). Rb1 did not affect eNOS protein expression (Fig. 2A and B, lower blots).

To see whether Rb1 actually activates NOS in HAECs, we measured NOS activity after 30 min of treatment with Rb1. As shown in Fig. 2C, Rb1 significantly increased NOS activity in HAECs.

PI3-kinase/Akt and MEK/ERK pathways are involved in eNOS phosphorylation and NO production

Previous studies have demonstrated that PI3-kinase/Akt and MEK/ERK pathways are two important signaling cascades mediating eNOS activation by many stimuli in vascular endothelial cells [6,7]. Therefore, we examined

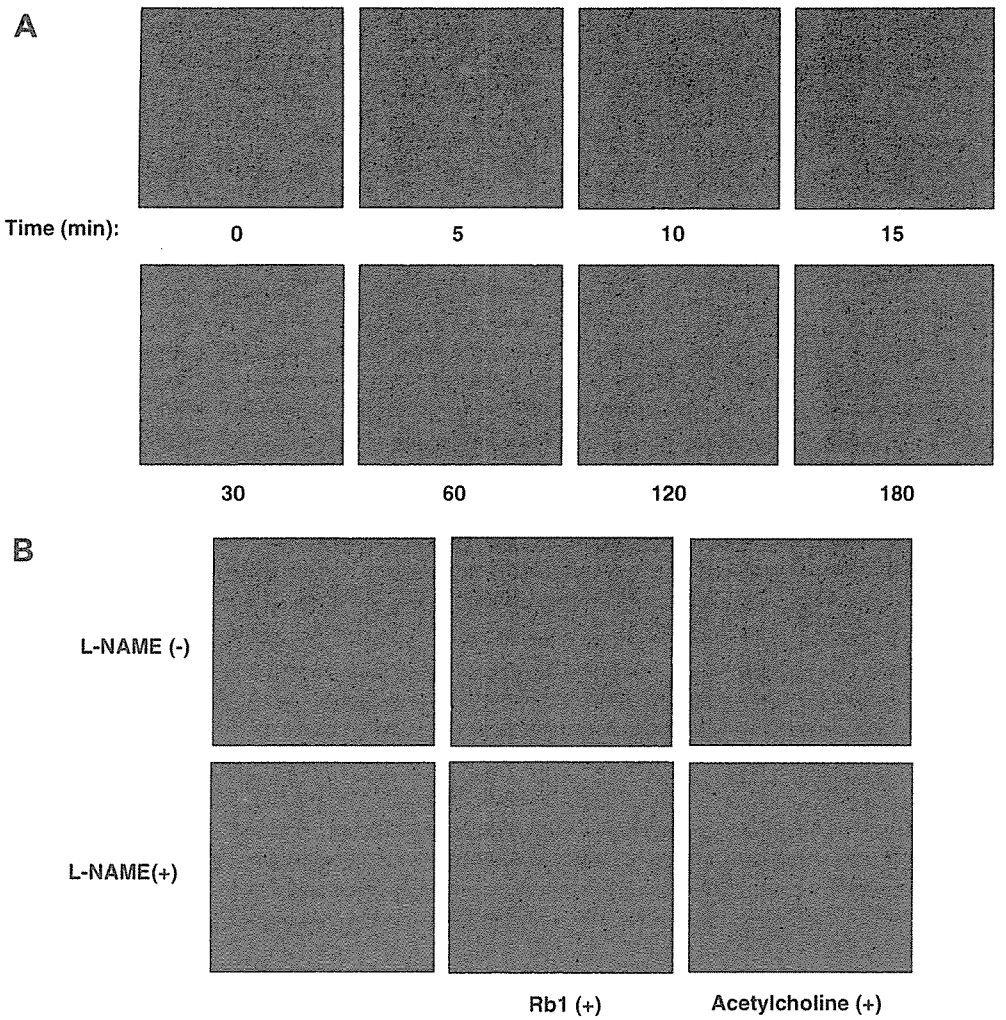

Fig. 1. Effect of Rb1 on production of NO. HAECs were starved and loaded with DAF-2 DA (5 μmol/L) as described in Materials and methods prior to treatment with either Rb1 (1 μmol/L) for 0, 5, 10, 15, 30, 60, 120, and 180 min (A) or acetylcholine (1 μmol/L) for 5 min (B). After Rb1 treatment, cells were fixed in 2% paraformaldehyde for 10 min at 4 °C and then viewed using a fluorescent microscope. Emission of green light (510 nm) from cells excited by light at 488 nm is indicative of NO production. In some groups of cells, L-NAME (0.5 mmol/L) was added 30 min before loading cells with DAF-2 DA (B). A representative time course experiment is shown for experiments that were repeated independently for three times. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

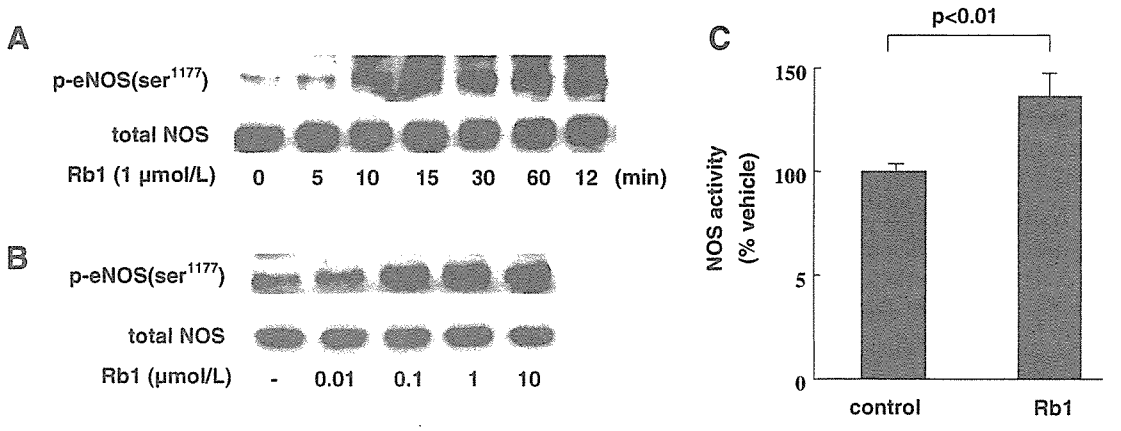

Fig. 2. Effects of Rb1 on eNOS phosphorylation and NOS activity. Phosphorylation of eNOS in HAECs. Starved HAECs were treated with the vehicle (0.01% DMSO) or Rb1 (1 μmol/L) for indicated times (A) or with various concentrations of Rb1 for 30 min (B). Western analysis performed to detect phospho-eNOS (Ser1177) and total eNOS. The experiments were repeated three times in triplicates, with equal result. NOS activity in HAECs homogenates. Rb1 (1 μmol/L) were added to the starved medium for 30 min, then activity of NOS was measured by the conversion of L-arginine to L-citrulline at 37 °C for 60 min (C). Histograms and error bars represent means ± SEM of four independent experiments performed in duplicate. $^{*}P < 0.01$ vs control.

whether Rb1 activated Akt and ERK1/2. We used phospho-specific antibodies to evaluate the ability of Rb1 to stimulate phosphorylation of Akt and ERK1/2 in HAECs. Rb1 rapidly increased phosphorylation of Akt (Ser473) and ERK1/2 (Fig. 3A, upper blots) in HAECs > 5 min after the addition of Rb1. Maximal phosphorylation was attained at 30 min in Akt and at 15 min in ERK1/2. The relative magnitude of the Rb1 response falls subsequently but is still significantly greater than control after 120 min of Rb1 treatment. Rb1 did not affect total Akt and ERK protein expression (Fig. 3A, lower blots).

We next examined the rapid phosphorylation of eNOS at Ser1179 by Rb1 either in the absence or presence of PI3 kinase inhibitor wortmannin, and Akt inhibitor SH-5 or MEK (ERK kinase) inhibitor PD98059. As shown in Fig. 3B, the rapid eNOS phosphorylation was abolished by pretreatment of cells with wortmannin (5 μmol/L) or SH-5, and partially attenuated by MEK inhibitor PD98059 (10 μmol/L). NO production viewed by fluorescent microscopy showed the similar inhibition by these inhibitors (Fig. 3C). These results suggest that acute activation of eNOS and NO production by Rb1 were mediated through activation of PI3-kinase/Akt and ERK1/2.

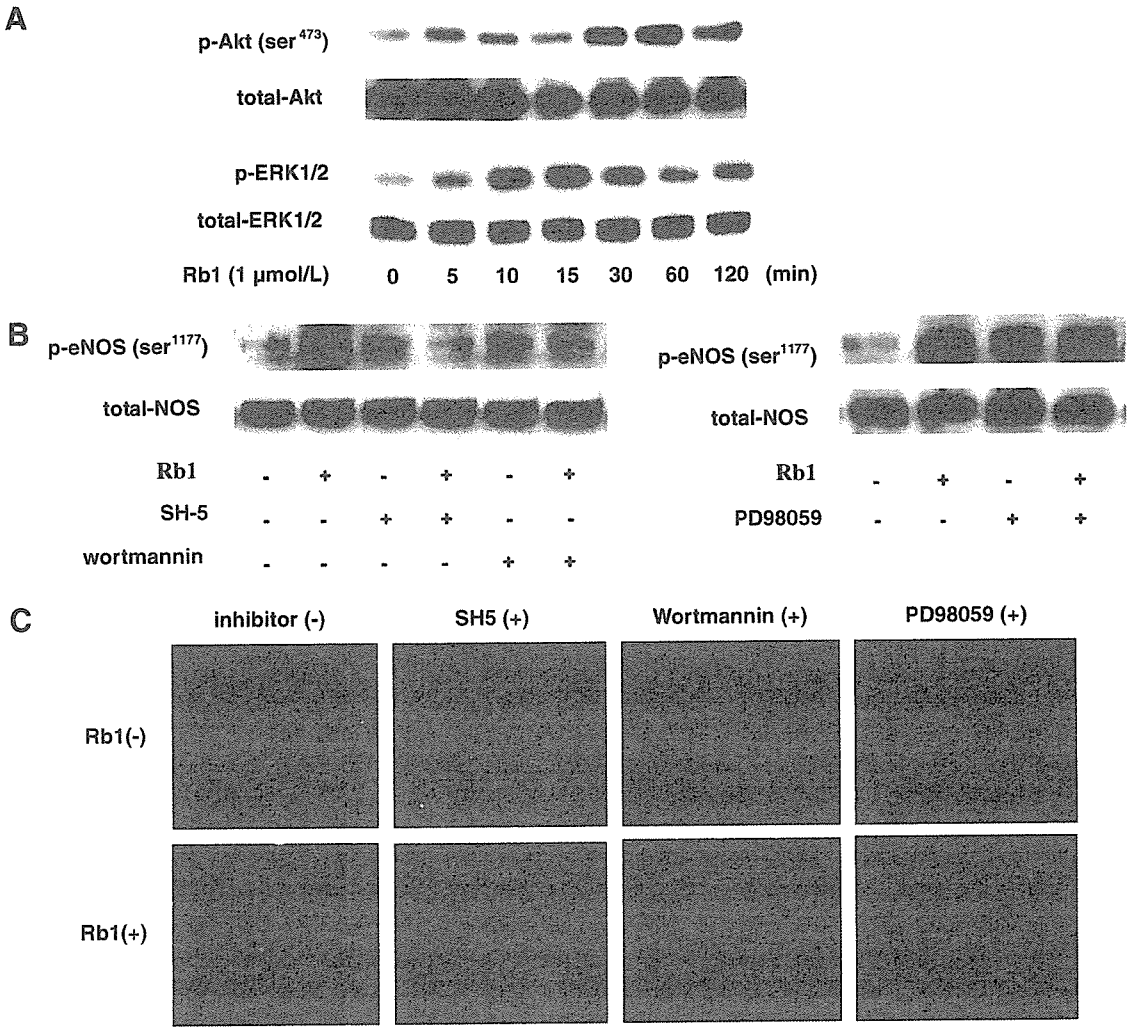

Fig. 3. Effects of inhibitors for PI3kinae/Akt or MEK (ERK kinase) on eNOS phosphorylation and NO production. Starved HAECs were treated with the vehicle (0.01% DMSO) or Rb1 (1 μmol/L) for indicated times (A). In some groups, cells were pretreated with SH-5 (10 μmol/L), wortmannin (5 μmol/L) or PD98059 (10 μmol/L) for 1 h, then cells were treated without or with Rb1 (1 μmol/L) for 30 min (B). Cell lysates were analyzed by Western blot as described in Materials and methods. Anti-phospho-Akt (Ser473) antibody and anti-Akt antibody; anti-phospho-ERK1/2 antibody and anti-ERK1/2 antibody (A), anti-phospho-eNOS (Ser1177) antibody and anti-eNOS antibody (B) were used for western blot analysis. Experiments were repeated three times, with equivalent result. Starved cells were loaded with DAF-2 DA as described in Materials and methods before treatment with Rb1 (1 μmol/L). In some groups of cells, SH-5 (10 μmol/L), wortmannin (5 μmol/L) or PD98059 (10 μmol/L) were added 60 min before cells were loaded with DAF-2 DA. After Rb1 treatment, cells were fixed in 2% paraformaldehyde for 10 min at 4 °C and then viewed using a fluorescent microscope (C). Emission of green light (510 nm) from cells excited by light at 488 nm is indicative of NO production. A representative set of experiments is shown for experiments that were repeated independently three times. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

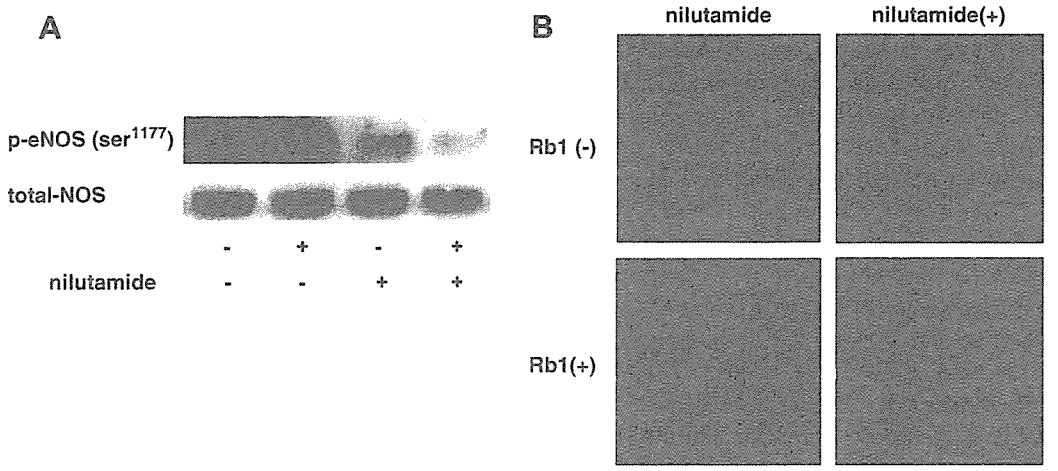

Fig. 4. Effects of nilutamide, an antagonist of androgen receptor, on eNOS phosphorylation and NO production. HAECs were starved 6 h and then treated without or with Rb1 (1 μmol/L) for 30 min. Some groups of cells were pre-treated with androgen receptor agonist nilutamide (10 μmol/L) for 1 h. Cell lysates were then subjected to immunoblotting as described in Materials and methods. The experiments were repeated three times in triplicates, with equal results. Starved cells were loaded with DAF-2 DA as described in Materials and methods before treatment with Rb1 (1 μmol/L). In some groups of cells, nilutamide (10 μmol/L) were added 60 min before cells were loaded with DAF-2 DA. After Rb1 treatment, cells were fixed in 2% paraformaldehyde for 10 min at 4 °C and then viewed using a fluorescent microscope (B). Emission of green light (510 nm) from cells excited by light at 488 nm is indicative of NO production. The experiments were repeated independently three times with equal results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Rb1-induced eNOS phosphorylation is inhibited by androgen receptor antagonist

Increasing evidence shows that activation of the steroid hormone receptor such as estrogen receptor (ER) lead to NO production and vasodilation within minutes by non-transcriptional pathways. Ginsenosides have steroidal skeleton structure and can act as an agonist for steroid hormones receptor. To see whether steroid hormone receptors were involved in acute activation of eNOS to produce NO in HUACs by Rb1, we examined the effects of the androgen receptor antagonist nilutamide and estrogen receptor antagonist ICI182780. Representative western blots obtained using anti-phospho-eNOS (Ser1177) antibody and anti-eNOS antibody are shown in Fig. 4A. The Rb1-induced eNOS phosphorylation (Ser1177) was inhibited by the androgen receptor antagonist nilutamid (10 μmol/L) In addition, NO production was diminished to the baseline level in the presence of nilutamid (Fig. 4B). However, the Rb1-induced eNOS phosphorylation (Ser1177) and NO production were unaffected by an estrogen receptor (ER) antagonist ICI182780 (10 μmol/L) (data not shown).

Discussion

We have shown that purified Rb1 rapidly stimulates production of NO in HAECs > 15 min after treatment. Maximal stimulation of NO production was obtained at 30 min. The increase in NO production was abrogated by the addition of eNOS inhibitor, L-NAME. It is generally well known that eNOS is tightly regulated not only at the transcriptional level but also by several post-transcriptional mechanisms [8]. The enhanced phosphorylation at Ser1177 leads to increased eNOS activity. In our experiments, Rb1 induced rapid phosphorylation of eNOS at Ser1177 > 10 min after Rb1 treatment. Maximal eNOS phosphorylation by Rb1 was observed from 30 to 60 min of incubation. NOS activity was also increased by the addition of Rb1 in HAECs. Taken together, our results suggest that the acute effect on NO production in HAECs is attributable to rapid phosphorylation of eNOS at Ser1177. NO produced by eNOS is a fundamental determinant of cardiovascular homeostasis responsible for regulating systolic blood pressure, vascular remodeling and angiogenesis. It is possible to consider that Rb1, a major active component of ginseng could be a candidate responsible for the antihypertensive effects of ginseng previously reported [1,2].

Recent studies have revealed that PI3-kinase/Akt and MEK/ERK1/2 pathways are crucial regulator in cell proliferation, cell-cycle progression, and mediator of cellular survival. Both of them also contribute to enhanced phosphorylation of eNOS at Ser1177/1179 and production of NO [6,7]. The present study showed that Rb1 also stimulated the phosphorylation of Akt (Ser473) and ERK1/2 (Thr202/Thr204) in HAECs. Rb1-induced eNOS phosphorylation was prevented by inhibitors for PI3-kinase/Akt or MEK (ERK kinase). Our data suggest that the activation of PI3-kinase/Akt and MEK/ERK-mediated pathways are involved in the regulation of acute eNOS phosphorylation by ginsenoside Rb1 in HAECs.

Another interesting finding is that acute phosphorylation of eNOS by Rb1 was abolished by an antagonist for androgen receptor. Recent studies have shown Rb1 acts as a phytoestrogen in MCF-7 human mammary carcinoma

cells [9]. However, in HAECs, Rb1-induced eNOS phosphorylation was not prevented by an antagonist for estrogen receptor (data not shown). It is known that testosterone prevents coronary artery disease, and lower testosterone level is a risk factor for ischemic heart disease in men [10–12]. Recent studies revealed that endothelial NO has antiatherosclerotic properties, such as inhibition of platelet aggregation, leukocyte adhesion, smooth muscle cell proliferation, and expression of genes involved in atherosclerosis [4]. Together with these observations, our results that Rb1 induced eNOS phosphorylation has been abolished by the androgen receptor antagonist will be the beginning of the experimental analyses at cellular levels and may provide a clue for better understanding of the mechanisms by which androgens exert their action for preventing coronary artery disease. Further studies are required for elucidation.

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References

[1] K.H. Han, S.C. Choe, H.S. Kim, D.W. Sohn, K.Y. Nam, B.H. Oh, M.M. Lee, Y.B. Park, Y.S. Choi, J.D. Seo, Y.W. Lee, Effect of red ginseng on blood pressure in patients with essential hypertension and white coat hypertension, Am. J. Chin. Med. 26 (1998) 199–209.

[2] N.D. Kim, S.Y. Kang, V.B. Schini, Ginsenosides evoke endothelium-dependant vascular relaxation in rat aorta, Gen. Pharmacol. 25 (1994) 1071–1077.

[3] N.D. Kim, S.Y. Kang, J.H. Park, V.B. Schini-Kerth, Ginsenoside Rg3 mediates endothelium-dependent relaxation in response to ginsenosides in rat aorta: role of K^+ channels, Eur. J. Pharmacol. 367 (1999) 41–49.

[4] A.G. Herman, S. Moncada, Therapeutic potential of nitric oxide donors in the prevention and treatment of atherosclerosis, Eur. Heart J. 26 (2005) 1945–1955.

[5] H. Kojima, N. Nakatsubo, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata, T. Nagano, Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins, Anal. Chem. 70 (1998) 2446–2453.

[6] X. Peng, S. Haldar, S. Deshpande, K. Irani, D.A. Kass, Wall stiffness suppresses Akt/eNOS and cytoprotection in pulse-perfused endothelium, Hypertension 41 (2003) 378–381.

[7] D. Feliers, X. Chen, N. Akis, G.G. Choudhury, M. Madaio, B.S. Kasinath, VEGF regulation of endothelial nitric oxide synthase in glomerular endothelial cells, Kidney Int. 68 (2005) 1648–1659.

[8] I. Fleming, R. Busse, Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase, Am. J. Physiol. Regul. Integr. Comp. Physiol. 284 (2003) R1–R12.

[9] J. Cho, W. Park, S. Lee, W. Ahn, Y. Lee, Ginsenoside-Rb1 from Panax ginseng C.A. Meyer activates estrogen receptor-alpha and -beta, independent of ligand binding, J. Clin. Endocrinol. Metab. 89 (2004) 3510–3515.

[10] G.B. Phillips, B.H. Pinkernell, T.Y. Jing, The association of hypotesteronemia with coronary artery disease in men, Arterioscler. Thromb. 14 (1994) 701–706.

[11] C.M. Webb, J.G. McNeill, C.S. Hayward, D. De Zeigler, P. Collins, Effects of testosterone on coronary vasomotor regulation in men with coronary heart disease, Circulation 100 (1999) 1690–1696.

[12] F.C. Wu, A. von Eckardstein, Androgens and coronary artery disease, Endocr. Rev. 24 (2003) 183–217.

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Gas6/Axl-PI3K/Akt pathway plays a central role in the effect of statins on inorganic phosphate-induced calcification of vascular smooth muscle cells

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Abstract

Apoptosis is essential for the initiation and progression of vascular calcification. Recently, we showed that *3-hydroxy-3-methylglutaryl* (HMG) CoA reductase inhibitors (statins) have a protective effect against vascular smooth muscle cell calcification by inhibiting apoptosis, where growth arrest-specific gene 6 (Gas6) plays a pivotal role. In the present study, we clarified the downstream targets of Gas6-mediated survival signaling in inorganic phosphate (Pi)-induced apoptosis and examined the effect of statins. We found that fluvastatin and pravastatin significantly inhibited Pi-induced apoptosis and calcification in a concentration-dependent manner in human aortic smooth muscle cells (HASMC), as was found with atorvastatin previously. Gas6 and its receptor, Axl, expression were downregulated in the presence of Pi, and recombinant human Gas6 (rhGas6) significantly inhibited apoptosis and calcification in a concentration-dependent manner. During apoptosis, Pi suppressed Akt phosphorylation, which was reversed by rhGas6. Wortmannin, a specific *phosphatidylinositol 3-OH kinase* (PI3K) inhibitor, abolished the increase in Akt phosphorylation by rhGas6 and eliminated the inhibitory effect of rhGas6 on both Pi-induced apoptosis and calcification, suggesting that PI3K-Akt is a downstream signal of the Gas6-mediated survival pathway. Pi reduced phosphorylation of Bcl2 and Bad, and activated caspase 3, all of which were reversed by rhGas6. The inhibitory effect of statins on Pi-induced apoptosis was accompanied by restoration of the Gas6-mediated survival signal pathway: upregulation of Gas6 and Axl expression, increased phosphorylation of Akt and Bcl2, and inhibition of Bad and caspase 3 activation. These findings indicate that the Gas6-mediated survival pathway is the target of statins' effect to prevent vascular calcification.

Keywords: Calcification; Apoptosis; Gas6; Axl; Akt; Bcl2

1. Introduction

Vascular calcification, such as coronary and aortic calcification, is clinically important in the development of cardiovascular disease (Eggen, 1968). Two distinct forms of vascular calcification are well recognized. One is medial calcification, which occurs between the cell layers of smooth muscle cells and is related to aging, diabetes and chronic renal failure (Neubauer, 1971; Goodman et al., 2000). The other is atherosclerotic calcification, which occurs in the intima during the development of atheromatous disease (Wexler et al., 1996). In diabetic patients, medial calcification has been shown to be a strong independent predictor of cardiovascular mortality (Everhart et al., 1988).

We recently demonstrated that atorvastatin prevented inorganic phosphate (Pi)-induced calcification by inhibiting apoptosis, one of the important processes regulating calcification. This was mediated by growth arrest-specific gene 6 (Gas6), a vitamin K-dependent protein (Son et al., 2006). Gas6 binds to Axl, the predominant receptor for Gas6, on the cell surface and transduces the signal by Axl autophosphorylation (Mark et al., 1996). Gas6-Axl interaction has been shown to be implicated in the regulation of multiple cellular functions (Yanagita et al., 2001; Goruppi et al., 1996; Nakano et al., 1997; Fridell et al., 1998). Especially, they are known to protect a range of cell types

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