

Improvement of Endothelial Function and Insulin Sensitivity With Vitamin C in Patients With Coronary Spastic Angina

Possible Role of Reactive Oxygen Species

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- OBJECTIVES** This study was designed to examine the effect of antioxidant supplementation on the endothelial function and insulin sensitivity in patients with coronary spastic angina (CSA).
- BACKGROUND** Insulin resistance may play a key role in coronary heart disease, and there is a possible link between acetylcholine-induced coronary vasoconstriction and hyperinsulinemia in patients with CSA. Endothelial dysfunction is present in the systemic arteries in CSA patients, and reactive oxygen species may cause inactivation of nitric oxide in these patients.
- METHODS** We measured flow-mediated dilation of the brachial artery using ultrasound technique in 22 patients with CSA and 20 control subjects. We also evaluated glucose tolerance using a 75-g oral glucose tolerance test and insulin sensitivity using steady-state plasma glucose (SSPG) methods in the same patients.
- RESULTS** The incidence of impaired glucose tolerance was higher in the CSA group than in the control group. Vitamin C infusion augmented flow-mediated dilation and decreased SSPG levels in the CSA group (from $3.27 \pm 0.77\%$ to $7.00 \pm 0.59\%$ [$p < 0.001$ by analysis of variance (ANOVA)] and from 177.3 ± 13.3 to 143.1 ± 14.9 mg/dl [$p = 0.047$ by ANOVA], respectively) but not in the control group (from $6.47 \pm 0.66\%$ to $6.80 \pm 0.60\%$ and from 119.8 ± 11.7 mg/dl to 118.1 ± 11.3 mg/dl, respectively). The steady-state plasma insulin levels were not affected by vitamin C infusion in either group.
- CONCLUSIONS** Vitamin C improves both endothelial function and insulin sensitivity in patients with CSA. Thus, reactive oxygen species and/or decreased nitric oxide bioactivity may play an important role in the genesis of both endothelial dysfunction and insulin resistance in patients with CSA. (J Am Coll Cardiol 2000;35:1860–6) © 2000 by the American College of Cardiology

There is increasing evidence that insulin resistance plays an important role in the pathogenesis of coronary heart disease (1,2). Glucose intolerance, hypertension, obesity and dyslipidemia have been suggested to promote atherosclerosis, with insulin resistance as the underlying intermediary (2,3). Recent studies suggested that insulin resistance may also be associated with coronary spasm (4–6). Endothelial dysfunction is an early and prominent event in atherogenesis (7). It has been shown that acetylcholine (ACh) causes vasodilation when the endothelium is intact by releasing nitric oxide (NO), whereas it causes vasoconstriction when the endothelium is removed or injured by directly acting on smooth

muscles (8). We have shown that ACh induces coronary spasm in patients with coronary spastic angina (CSA) and that both basal and NO activity are impaired in the coronary artery of these patients (9–11).

Insulin has been shown to cause endothelium-dependent vasodilation by releasing NO (12,13), and several studies have revealed that endothelium-dependent vasodilation is impaired in patients with insulin resistance (14,15). The decreased endothelium-dependent vasodilation observed in these patients may result from the inactivation of endothelium-derived NO by reactive oxygen species (16), and vitamin C, an antioxidant, is reported to improve endothelium-dependent vasodilation in these patients (17,18). Recently, noninvasive examination of endothelial function has become possible using flow-mediated endothelium-dependent dilation (FMD) of the brachial artery during reactive hyperemia (19–21), and we have shown that FMD of the brachial artery is impaired in

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Abbreviations and Acronyms

Ach	= acetylcholine
ANOVA	= analysis of variance
CSA	= coronary spastic angina
FMD	= flow-mediated dilation
HDL	= high-density lipoprotein
NO	= nitric oxide
OGTT	= oral glucose tolerance test
SSPG	= steady-state plasma glucose
SSPI	= steady-state plasma insulin

patients with CSA (22,23). There are no reports, however, whether FMD is improved in patients with CSA by vitamin C. There are also no data on the possible role of antioxidants in the genesis of insulin resistance.

The present study was designed to examine whether vitamin C improves endothelial dysfunction and insulin resistance in patients with CSA and without diabetes mellitus compared with controls.

METHODS

Study subjects. We studied 22 patients with CSA (mean age 55.6 ± 3.1 years, 13 men and 9 women) in whom episodes of spontaneous angina occurred at rest. All patients with CSA had angiographically normal coronary arteries and documented coronary spasm associated with ischemic ST segment changes after the intracoronary injection of Ach (9,10). The present study also included 20 control subjects (mean age 57.1 ± 1.8 years, 12 men and 8 women). These control subjects were selected to match the cardiovascular risk factors (age, gender, smoking status, body mass index, and hypertension) to those of the patients with CSA. The control subjects underwent coronary catheterization for evaluation of chest pain. They had angiographically normal coronary arteries and did not have coronary spasm after the intracoronary injection of Ach. Patients with diabetes mellitus, obesity (body mass index >26), familial hypercholesterolemia, previous myocardial infarction, congestive heart failure or other serious diseases were excluded. Written, informed consent was obtained from all subjects before the study. The study was in agreement with the guidelines approved by the ethics committee at our institution.

Oral glucose tolerance test. After the overnight fast, a 75-g oral glucose tolerance test (OGTT) was performed in all subjects. Blood samples were drawn in the fasting state, at 60 and 120 min after the glucose loading for determination of plasma glucose and insulin levels. The definition of glucose tolerance was based on the World Health Organization criteria (24).

Measurements of insulin sensitivity. Insulin sensitivity was estimated by the steady-state plasma glucose (SSPG) methods with the use of Sandostatin (25). Sandostatin, an analogue of somatostatin that inhibits the endogenous

secretion of insulin, glucagon and growth hormone, has a longer duration of action than somatostatin and is more potent than the natural compound (26). After the overnight fast, Novolin R 40 insulin was injected in a bolus (7.5 mU/kg) initially, and glucose (6 mg/kg/min) KCl (0.5 μ Eq/kg/min), Novolin R 40 insulin (0.77 mU/kg/min) and Sandostatin (150 μ g/2 h) were infused simultaneously for 2 h through an antecubital vein via a constant infusion pump. Blood samples were obtained at 0 and 120 min for the determination of plasma glucose and insulin. Steady-state plasma glucose and steady-state plasma insulin (SSPI) concentrations were obtained at 120 min. Under these steady-state conditions, plasma glucose was inversely correlated with the rate of insulin-mediated glucose disposal and was inversely proportional to insulin sensitivity (27).

Measurements of FMD of the brachial artery. The measurement of the brachial artery diameter was performed using the ultrasound technique as described in our previous studies and by others (19-23). The subjects lay quietly for at least 10 min before the first scan. The brachial artery was scanned in the antecubital fossa in a longitudinal fashion. Gain setting was optimized at the beginning of the study and was kept constant throughout the recording period. When a satisfactory transducer position was found, the arm remained at the same position throughout the study. The diameter of the brachial artery was measured from B-mode ultrasound images with a 7.5-MHz linear array transducer (SSH-160A; Toshiba, Tokyo, Japan). The flow velocity of the brachial artery was measured with a pulsed Doppler signal at a 70-degree angle to the vessel, with the range gate (1.5 mm) in the center of the artery. After the baseline measurements of the diameter and the flow velocity in the brachial artery, a blood-pressure cuff placed around the forearm was inflated with a pressure of 250 to 300 mm Hg. After 5 min, the cuff was deflated. During the cuff inflation and deflation, diameter and flow velocity were measured continuously. Images were recorded on a super-VHS videocassette recorder (BR-S 601M; Victor, Tokyo, Japan), and the brachial arterial diameters were measured from the tape with ultrasonic calipers. The measurements were performed by two independent observers who were blinded as to which group the individual belonged to or from which infusion the images had acquired.

In our study, the interobserver variability for the repeated measurements of resting arterial diameter was 0.05 ± 0.02 mm. The intraobserver variability for the repeated measurements of resting arterial diameter was 0.02 ± 0.02 mm. In a preliminary study, when these procedures were performed at the same time on two separate days in 20 volunteers, the average intrasubject test-retest difference for the measurements of the arterial diameter during the reactive hyperemia was 0.05 ± 0.04 mm.

Vitamin C infusion protocol. Vitamin C (ascorbic acid; Takeda, Osaka, Japan) was administered via the antecubital

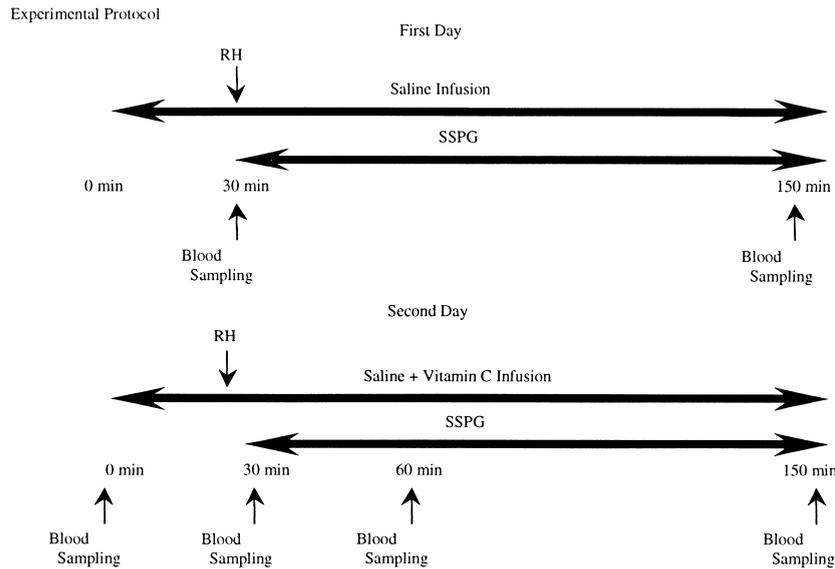


Figure 1. Schematic representation of the experimental protocol. RH = analysis of reactive hyperemia; SSPG = steady-state plasma glucose.

vein. Vitamin C was infused at a constant dose of 10 mg/min to limit the total vitamin C dose to <2,000 mg.

Experimental protocol. The experimental protocols were designed as two-day protocols (Fig. 1). On the first day, FMD was examined during saline infusion used as a placebo, and thereafter an insulin sensitivity test was performed in the morning in the fasting state. On the second day, FMD was examined 30 min after the initiation of saline plus vitamin C infusion. Then, the insulin sensitivity test was started sequentially under concomitant infusion of saline plus vitamin C. Blood sampling for concentration of vitamin C was done 0, 30 (at the start of the insulin sensitivity test), 60 and 150 min (at the termination of the insulin sensitivity test) after the initiation of saline plus vitamin C infusion.

All medications except sublingual nitroglycerin were withdrawn at least three days before the study. No patients had taken nitroglycerin within 6 h before the study. Blood pressure and heart rate were monitored during the study.

Analysis of blood samples. Plasma concentration of glucose was measured by the glucose oxidase method, and insulin by radioimmunoassay. Vitamin C concentration was measured by high-performance liquid chromatography (28).

Statistics analysis. The clinical characteristics were compared between the two groups by using the unpaired *t* test for continuous data and the chi-square test for group data. The changes in variables were assessed by two-way analysis of variance (ANOVA) with repeated measures followed by post-hoc testing with Scheffe’s test. Correlations with FMD, SSPG and vitamin C levels were examined using linear regression analysis. Statistical significance was defined as *p* < 0.05. Data were expressed as mean ± standard error of the mean.

RESULTS

Baseline characteristics and oral glucose tolerance test.

There were no differences in the baseline characteristics between the CSA group and the control group except for the incidence of impaired glucose tolerance and the plasma levels of high-density lipoprotein (HDL)-cholesterol, as shown in Table 1. The incidence of impaired glucose tolerance was higher in the CSA group than in the control group. The HDL cholesterol levels were lower in the CSA patients than in the control subjects.

Table 1. Patient Characteristics

	CSA (n = 22)	Control (n = 20)	p Values
Gender			0.800
Men	13 (59.1%)	12 (60.0%)	
Women	9 (40.9%)	8 (40.0%)	
Age, years	55.6 ± 3.1	57.1 ± 1.8	0.661
Smoker	13 (59.1%)	9 (45.0%)	0.361
IGT	9 (40.9%)	2 (10.0%)	0.023
Hypertension	5 (22.7%)	4 (20.0%)	0.830
Body mass index (kg/m ²)	23.6 ± 0.8	23.6 ± 0.9	0.980
Total cholesterol (mg/dl)	188.4 ± 6.6	198.1 ± 11.8	0.736
HDL cholesterol (mg/dl)	45.2 ± 3.0	55.1 ± 3.5	0.028
LDL cholesterol (mg/dl)	113.5 ± 7.0	118.3 ± 9.5	0.678
Triglyceride (mg/dl)	146.1 ± 17.2	123.4 ± 13.0	0.266

CSA = coronary spastic angina; HDL = high-density lipoprotein; IGT = impaired glucose tolerance; LDL = low-density lipoprotein.
 Values are expressed as mean ± SE.

Table 2. Oral Glucose Tolerance Test

	CSA (n = 22)	Control (n = 20)	p Values
Glucose			
Fasting (mg/dl)	91.8 ± 2.0	94.4 ± 2.6	0.798
60 min (mg/dl)	177.5 ± 9.8	142.3 ± 8.8	0.012
120 min (mg/dl)	135.3 ± 5.2	113.9 ± 6.8	0.013
Insulin			
Fasting (μU/ml)	9.2 ± 0.5	7.1 ± 0.4	0.001
60 min (μU/ml)	85.9 ± 12.0	53.1 ± 6.1	0.012
120 min (μU/ml)	56.6 ± 5.8	37.8 ± 4.5	0.015

CSA = coronary spastic angina.
Values are expressed as mean ± SE.

Plasma glucose and insulin responses to OGTT are shown in Table 2. The fasting insulin levels were higher in the CSA group than in the control group, whereas there was no difference between the two groups in the fasting glucose levels. The glucose levels at 60 and 120 min were higher in the CSA group than in the control group. The insulin levels at 60 and 120 min were also higher in the CSA group than in the control group.

The basal vitamin C levels were lower in the CSA group than in the control group, as shown in Table 3. The levels increased in both groups after vitamin C infusion, and there were no differences in the levels during the infusion time between the two groups.

Response of FMD to vitamin C. The baseline hemodynamics parameters were not different between the two groups. There was also no difference in the arterial diameter (control: 4.26 ± 0.14, CSA: 4.26 ± 0.17 mm, p = 0.979, NS), the blood flow or the increases in blood flow during reactive hyperemia between the two groups at the baseline. However, FMD was reduced in the CSA group at the baseline (3.27 ± 0.77 vs 6.47 ± 0.66%, p < 0.001).

Vitamin C infusion did not affect hemodynamics parameters, the arterial diameter, the blood flow or the increases in blood flow during reactive hyperemia. However, vitamin C infusion augmented FMD in the CSA group (from 3.27 ± 0.77% to 7.00 ± 0.59%, p < 0.001) but not in the control group (from 6.47 ± 0.66 to 6.80 ± 0.60%, p = 0.804, NS). The differences of FMD between the CSA group and the control group analyzed by two-way ANOVA were significant (p = 0.048). Those of FMD between the saline infusion and the saline plus vitamin C infusion analyzed by

Table 3. Plasma Vitamin C Concentration

	CSA (n = 22)	Control (n = 20)	p Values
0 min	34.6 ± 3.4	48.3 ± 4.5	0.023
30 min	180.6 ± 17.6	234.0 ± 23.3	0.092
60 min	245.9 ± 26.1	280.6 ± 26.1	0.365
150 min	355.6 ± 31.2	388.5 ± 28.4	0.752

CSA = coronary spastic angina.
Values (μmol/liter) are expressed as mean ± SE.

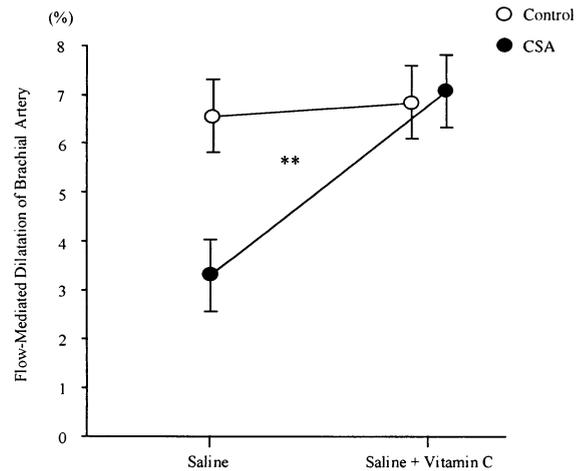


Figure 2. Effect of vitamin C on flow-mediated dilation in patients with coronary spastic angina and in control subjects. See text for details. Data are expressed as mean value ± SE. **Significant effect of vitamin C infusion on the CSA group compared with the control group, p < 0.001 by ANOVA. CSA = coronary spastic angina.

two-way ANOVA were also significant (p < 0.001). The effect of vitamin C on FMD in the CSA group was significantly different from that in the control group, as shown in Figure 2 (p < 0.001, by ANOVA).

Response of SSPG to vitamin C. There were no changes in hemodynamics during the insulin sensitivity test. The baseline SSPG levels were higher in the CSA group than in the control group (177.3 ± 13.3 vs. 119.8 ± 11.7 mg/dl, p < 0.001). There was no difference in the baseline SSPI levels between the two groups (38.7 ± 4.0 vs. 35.2 ± 3.2 U/ml, p = 0.494, NS).

Vitamin C infusion decreased the SSPG levels in the CSA group (from 177.3 ± 13.3 to 143.1 ± 14.9 mg/dl, p = 0.013) but not in the control group (from 119.8 ± 11.7 mg/dl to 118.1 ± 11.3 mg/dl, p = 0.967, NS). The differences of SSPG between the CSA group and the control group analyzed by two-way ANOVA were significant (p = 0.017). Those of SSPG between the saline infusion and the saline plus vitamin C infusion analyzed by two-way ANOVA were also significant (p = 0.008). The effect of vitamin C on the SSPG levels in the CSA group was significantly different from those in the control group, as shown in Figure 3 (p = 0.047, by ANOVA). The SSPI levels were not affected by vitamin C infusion in either group.

Correlation between FMD and vitamin C levels and that between SSPG and vitamin C levels. There was a positive correlation between FMD and the vitamin C levels at baseline (r = 0.472, p < 0.001), as shown in Figure 4A. There was a negative correlation between the SSPG levels and the vitamin C levels at baseline (r = -0.469, p = 0.002) (Fig. 4B). Both correlations disappeared after vita-

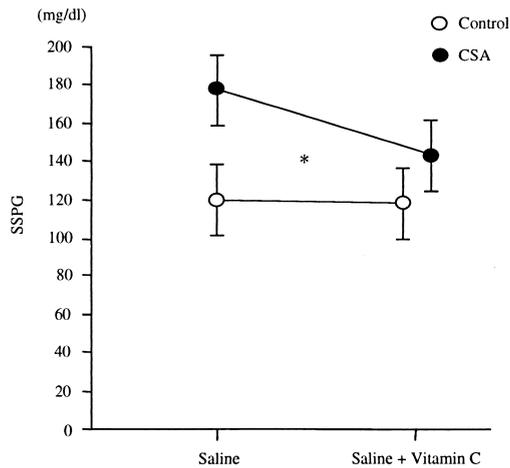


Figure 3. Effect of vitamin C on steady-state plasma glucose in patients with coronary spastic angina and in control subjects. See text for details. Data are expressed as mean value \pm SE. *Significant effect of vitamin C infusion on the CSA group compared with the control group, $p < 0.05$ by ANOVA. CSA = coronary spastic angina; SSPG = steady-state plasma glucose.

min C administration ($r = 0.113$, $p = 0.576$, NS, $r = -0.381$, $p = 0.073$, NS, respectively).

Correlation between SSPG and FMD. There was a negative correlation between the SSPG levels and FMD ($r = -0.492$, $p = 0.002$), as shown in Figure 5. The correlation disappeared after vitamin C infusion ($r = 0.008$, $p = 0.971$, NS).

DISCUSSION

Recent studies have shown that FMD of the brachial artery during reactive hyperemia is endothelium (20-23) and NO dependent (19). The present study showed that FMD of the brachial artery was decreased in the patients with CSA compared with controls. The study also showed that the plasma levels of vitamin C were decreased, and there was a positive correlation between the vitamin C levels and FMD at baseline. Flow-mediated dilation or the endothelial dysfunction was improved, and the significant correlation between FMD and plasma vitamin C levels disappeared after treatment of vitamin C, an antioxidant, in these patients. We have shown previously that endothelium-dependent dilations of both the coronary artery and the brachial artery were impaired in patients with CSA and that these impairments were improved by treatment with antioxidants, vitamin C and vitamin E in these patients (22,29). The present study, thus, confirms the results of our previous studies.

We also demonstrate that insulin sensitivity as assessed by the levels of SSPG and levels of fasting plasma insulin was impaired in patients with CSA compared with controls. These results are in agreement with those of the previous studies (4-6). In addition, the present study showed that

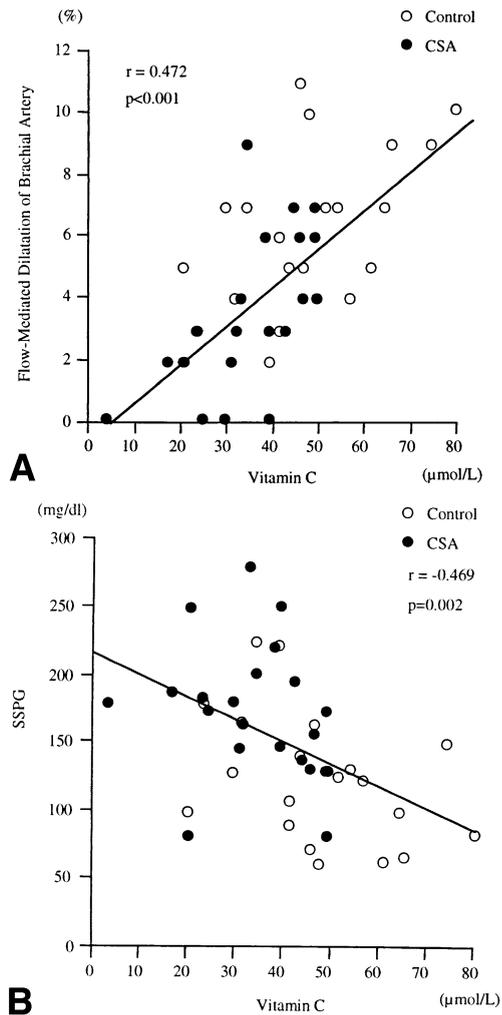


Figure 4. Correlation between: (A) plasma vitamin C concentration and flow-mediated dilation, and that between (B) vitamin C concentration and steady-state plasma glucose before vitamin C infusion. SSPG = steady-state plasma glucose.

there was a negative correlation between the SSPG and the vitamin C levels at baseline, and the elevated SSPG levels or the impairment of insulin sensitivity was improved along with FMD or a marker of endothelial function by the vitamin C supplementation. There was a negative correlation between the SSPG levels and FMD at baseline. However, the correlation disappeared after administration of vitamin C, probably because vitamin C eliminated the effects of reactive oxygen species. Thus, the present study shows for the first time that there is a close correlation between endothelial function and insulin sensitivity and that reactive oxygen species play an important role in the genesis of insulin resistance as well as endothelial dysfunction.

Increased reactive oxygen species inactivate endothelial NO and lead to endothelial dysfunction (16,30). Endothelium plays an important role in the regulation of blood flow to insulin-sensitive tissues, and insulin induces endothelium-dependent

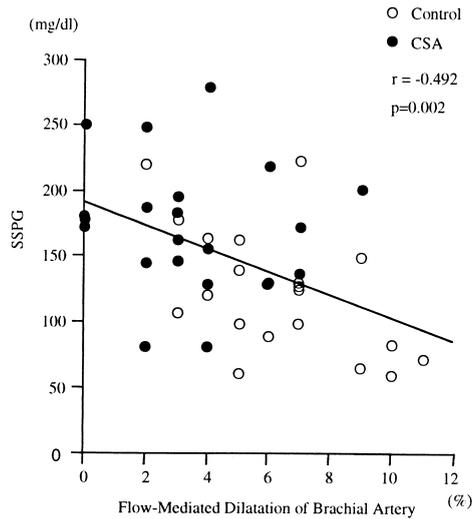


Figure 5. Correlation between flow-mediated dilation and steady-state plasma glucose before vitamin C infusion. SSPG = steady-state plasma glucose.

vasodilation (12,13). Impaired insulin-induced increase in skeletal muscle blood flow has been reported in insulin resistance states, including obesity, hypertension and type 2 diabetes mellitus (31). It is thus possible that reactive oxygen species cause endothelial dysfunction, which may contribute to insulin resistance through impaired delivery of insulin to the insulin-sensitive tissues such as skeletal muscles. Other studies, however, have reported that insulin resistance may occur in the absence of reduced blood flow in skeletal muscles (32,33). It is possible that reactive oxygen species may cause flow-independent insulin resistance by inhibiting insulin receptor signaling.

The sources of increased reactive oxygen species are not clear. We and others have shown that cigarette smoking is a highly significant risk factor for coronary spasm (34-37) and that cigarette smoke extracts contain large amounts of reactive oxygen species that cause endothelial dysfunction (30,38). We have also shown that patients with CSA are significantly associated with mutations of endothelial NO synthase gene (36,37), which may lead to reduced endothelial production of NO and thus reduced scavenging of reactive oxygen species. These factors may play important roles in the production of increased reactive oxygen species in patients with CSA.

High-density lipoprotein-cholesterol is known as an antioxidant (39), and its plasma levels were decreased in the CSA group compared with the control group in the present study. We previously reported that, in the patients with CSA, the oxidative stress increased compared with the control subjects (22). Thus, there may be a close relationship between the increased reactive oxygen species and HDL cholesterol levels in patients with CSA.

Recently, ascorbic acid has been shown to potentiate NO synthesis in endothelial cells through improvement of cel-

lular redox state or glutathione metabolism (20,40,41). Furthermore, Jackson et al. reported that normal extracellular concentrations of ascorbic acid (30 to 150 $\mu\text{mol/liter}$) are not likely to prevent the interaction of NO with superoxide under physiological condition (41). In the present study, the plasma vitamin C concentrations were lower in the patients with CSA than in the control subjects. Thus, another possible mechanism of the present results may be that a deficiency of vitamin C decreased NO synthesis in patients with CSA.

Study limitations. In the present study, we examined FMD of the brachial artery as a marker of endothelial function. However, endothelial dysfunction at the arteriolar and capillary levels may be metabolically more important in relation to insulin resistance (42). However, atherosclerosis is a disease of large conduit arteries and insulin resistance is associated with risk factors of atherosclerosis, including hypertension, dyslipidemia, type 2 diabetes and obesity (43). It is thus quite probable that the endothelium of both large conduit arteries and resistance vessels are impaired by increased reactive oxygen species in patients with coronary risk factors, including those with CSA.

In conclusion, both FMD of the brachial artery and insulin sensitivity as assessed by SSPG levels are impaired in patients with CSA, and these impairments are improved by the administration of vitamin C. The results indicate that there is a close relationship between endothelial function and insulin sensitivity and that increased reactive oxygen species and/or decreased NO bioactivity may play an important role in the genesis of both endothelial dysfunction and insulin resistance in patients with CSA.

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