Endothelium-dependent vasodilation in the brachial artery is impaired in smokers: effect of vitamin C

TAKESHI MOTOYAMA, HIROAKI KAWANO, KIYOTAKA KUGIYAMA, OSAMU HIRASHIMA, MASAMICHI OHGUSHI, MICHIHIRO YOSHIMURA, HISAO OGAWA, AND HIROYUJI YASUE

Division of Cardiology, Kumamoto University School of Medicine, Kumamoto City 860, Japan

Motoyama, Takeshi, Hiroaki Kawano, Kiyotaka Kugiyaama, Osamu Hirashima, Masamichi Ohgushi, Michihiro Yoshimura, Hisao Ogawa, and Hirofumi Yasue. Endothelium-dependent vasodilation in the brachial artery is impaired in smokers: effect of vitamin C. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H1644–H1650, 1997.—Cigarette smoking has been shown to cause endothelial dysfunction. To examine the effects of vitamin C and cigarette smoking on endothelium-dependent vasodilation, we measured the luminal diameter and flow velocity of the brachial arteries at rest, during reactive hyperemia following transient arterial occlusion, and after sublingual nitroglycerin (0.3 mg) in smokers (n = 20) and nonsmokers (n = 20) with high-resolution ultrasound after infusion of saline or saline plus vitamin C (10 mg/min for 20 min). We also performed the same study in smokers (n = 15) before and after smoking. In addition, we measured the serum levels of vitamin C and the plasma levels of thiobarbituric acid-reactive substances (TBARS) as an index of lipid peroxidation. The smokers had lower vitamin C levels, higher TBARS levels, and showed impairment of flow-dependent vasodilation (5.3 ± 1.9 vs. 9.2 ± 1.5%, P < 0.0001) compared with nonsmokers. Vitamin C administration improved the impairment of flow-dependent vasodilation (5.3 ± 1.9 to 9.0 ± 3.2%, P < 0.001) and decreased TBARS in smokers but not in nonsmokers. Furthermore, cigarette smoking acutely worsened the impairment of flow-dependent vasodilation (5.4 ± 1.8 to 1.5 ± 1.3%, P < 0.01) and increased TBARS. We conclude that 1) endothelium-dependent vasodilation in the brachial arteries is impaired in smokers and this impairment is improved by vitamin C administration in association with a decrease in TBARS and 2) cigarette smoking produces acute impairment of endothelium-dependent vasodilation in smokers in association with an increase in TBARS.

oxidative stress; flow-mediated vasodilation; high-resolution ultrasound; antioxidant

ENDOTHELIAL DYSFUNCTION occurs at an early stage of atherosclerosis in human systemic arteries (24). Cigarette smoking, which is a major risk factor for coronary artery disease (10, 28), has been shown to be associated with endothelial dysfunction (5, 31). Although the precise mechanism of the smoking-induced endothelial dysfunction is unknown, recent clinical and experimental observations (12, 17, 18, 22) strongly suggest a potential role of oxygen-derived free radicals. Vitamin C has a broad spectrum of antioxidant activities because of its ability to react with numerous aqueous free radicals and reactive oxygen species and effectively protects lipids in human plasma against peroxidative damage (7). In addition, epidemiologic studies (25) have shown that smokers have significantly lower plasma levels of antioxidant nutrients, such as vitamins C and E, compared with nonsmokers. Recently, it has been reported (9) that vitamin C improves endothelial dysfunction of forearm resistance vessels in chronic smokers. Acute cigarette smoking causes vasoconstriction of epicardial coronary arteries and increases coronary resistance vessel tone (15, 20). However, the mechanism of smoking-induced vasoconstriction has not been elucidated. Cigarette smoke has been reported (3, 19) to contain nicotine and large amounts of free radicals, such as superoxide anion and hydroxyl radicals, and various other chemicals that directly or indirectly cause short-term effects on artery tone and hemodynamic variables (15, 16, 18, 20). Blood flow or shear stress is shown to play an important role in the endothelium-dependent regulation of vessel tone. Recently, high-resolution ultrasound has been used to assess the flow-mediated, endothelium-dependent vasodilation of the brachial artery in humans (1, 5, 11, 16).

The present study was designed to examine the effects of vitamin C infusion on flow-mediated, endothelium-dependent vasodilation of the brachial artery in smokers and nonsmokers with the use of ultrasound techniques together with the measurement of plasma levels of thiobarbituric acid-reactive substances (TBARS) as an index of oxidative stress.

METHODS

Study Subjects

We studied 40 healthy men between the ages of 26 and 35 yr (mean age: 30.1 ± 4.5 yr) and 20 current smokers (mean age: 30.1 ± 6.7 yr) and 20 age-matched nonsmokers (mean age: 30.0 ± 3.1 yr) who did not have regular exposure to environmental tobacco smoke. In smokers, the daily intake of cigarettes ranged from 15 to 40 (mean: 18 ± 5), and they had smoked at least 15 cigarettes/day for ≥7 yr. None of the study subjects had hypertension, diabetes mellitus, hypercholesterolemia, obesity, or other risk factors for coronary artery disease. There were no significant differences in the clinical characteristics between the smokers and nonsmokers, as shown in Table 1. Written informed consent was obtained from all study subjects before the study. The study was in agreement with the guidelines approved by the ethics committee at our institution.

Ultrasound Studies

The vasodilator responses in the brachial arteries were measured by the ultrasound technique validated previously by our study and others (1, 5, 6, 11). Briefly, the diameter of the brachial artery was measured from B-mode ultrasound images with the use of a 7.5-MHz linear-array transducer (model SSH-160A ultrasound system; Toshiba, Tokyo, Japan). The flow velocity of the brachial artery was measured with the use of a pulsed Doppler signal at a 70° angle to the vessel, with the range gate (1.5 mm) in the center of the artery. The

H1644 0363-6135/97 $5.00 Copyright © 1997 the American Physiological Society
Table 1. Clinical characteristics of study subjects

<table>
<thead>
<tr>
<th></th>
<th>Smokers</th>
<th>Non-smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>30.1 ± 6.7</td>
<td>30.0 ± 3.1</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>162.2 ± 41.6</td>
<td>165.5 ± 49.8</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>50.8 ± 10.4</td>
<td>53.1 ± 12.1</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>79.5 ± 12.1</td>
<td>51.1 ± 11.2</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22.1 ± 3.9</td>
<td>22.3 ± 4.1</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD for 20 male smokers and 20 male nonsmokers. HDL, high-density lipoprotein. There are no significant differences between smokers and nonsmokers.

brachial artery was scanned in the antecubital fossa in a longitudinal fashion. When a satisfactory transducer position was found, the surface of the skin was marked, and the arm remained in the same position throughout the study. Gain setting was optimized at the beginning of the study and was kept constant throughout the recording period. The subjects lay quietly for 10 min before the scan. After baseline measurements of the diameter and the flow velocity in the brachial artery were taken, a blood pressure cuff placed around the forearm was inflated with a pressure of 250–300 mmHg. After 4.5 min, the cuff was released. The diameter and flow velocity were continuously measured during cuff inflation and after cuff deflation. The flow-mediated dilator response was used as a measure of endothelium-dependent vasodilatation. In addition, the diameter was measured before and 3 min after sublingual nitroglycerin administration (300 μg). The response to nitroglycerin was used as a measure of endothelium-independent vasodilatation.

Images were recorded on a super-VHS videocassette recorder (BR-S601M; Victor, Tokyo, Japan), and brachial arterial diameters were measured from the tape with ultrasonic calipers by two observers who were blinded as to the protocols of the study and the subject grouping. The arterial diameter was measured at a fixed distance from anatomic markers such as a bifurcation, facial plane, or vein seen in cross section by point-to-point measurements. Measurements were taken from the anterior to the posterior interface between media and adventitia (M line) at end diastole, incident with the R wave on a continuously recorded electrocardiogram (1, 5, 6, 11, 29). Diameters at four cardiac cycles were analyzed for each scan, and the measurements were averaged. The diameter measurements for reactive hyperemia were taken 45–90 s after cuff deflation. The response of the vessel diameter to reactive hyperemia was expressed as a percent change relative to that just before cuff inflation. The response of the vessel diameter to nitroglycerin was expressed as a percent increase relative to that just before nitroglycerin administration. We measured Doppler flow and arterial diameter simultaneously. Blood flow was calculated by multiplying the velocity-time integral of the Doppler flow signal by heart rate and the vessel cross-sectional area of the artery measured at that time. The increase in brachial blood flow was calculated as a maximum flow recorded in the first 15 s after cuff deflation and was expressed as a percent change relative to that just before cuff inflation (1, 5, 6, 11).

In our studies, the interobserver variability for repeated measurement of resting arterial diameter was 0.1 ± 0.0 mm. The interobserver variability for repeated measurement of resting arterial diameter was 0.0 ± 0.1 mm. Furthermore, when these studies were performed at the same time on two separate days in 20 healthy volunteers, the difference in measurements of the percent increase in arterial diameter during reactive hyperemia between occasions and within patients was 1.4 ± 1.2%.

Study Protocol

Studies were performed in a quiet and temperature-controlled (22–24°C) room in the early morning at 7:00 AM while the study subjects were in the fasting state. No subjects were on any medication including vitamins, and all refrained from intake of caffeine-containing food or beverages for at least 12 h before the studies (26). All subjects abstained from smoking for at least 8 h before the studies. Heart rate and blood pressure were monitored continuously during the study period.

Protocol 1. On the first study day, measurements of flow-dependent vasodilatation and blood sampling for assay of TBARS were performed just after intravenous infusion of saline at a rate of 10 ml/min for 20 min, and measurements were repeated just after intravenous infusion of saline plus vitamin C at a rate of 10 ml/min (10 mg/min for 20 min) in both groups of subjects. After another 5 min of rest, nitroglycerin-induced vasodilatation was measured, and the data were analyzed as the responses to nitroglycerin after vitamin C infusion.

Protocol 2. This protocol was designed to examine the acute effects of cigarette smoking on endothelium-dependent vasodilatation in the smokers. Seven days after vitamin C administration, by which time the serum level of vitamin C had returned to the baseline level, 15 smokers smoked one filtered cigarette over 3–5 min (nicotine content was 0.9 mg). Measurements of flow-dependent vasodilatation and blood sampling for assay of TBARS were performed before and 10 min after cigarette smoking after intravenous infusion of saline. After another 5 min of rest, nitroglycerin-induced vasodilatation was measured, and the data were analyzed as the responses to nitroglycerin after cigarette smoking. On the next day, the acute effect of the cigarette smoking on flow-dependent vasodilatation was again examined after intravenous infusion of saline plus vitamin C (10 mg/min for 20 min).

The nitroglycerin-induced vasodilatation at the baseline (before vitamin C infusion) was examined after intravenous infusion of saline alone at intervals of 7 days after vitamin C infusion in all study subjects. Furthermore, this baseline vasodilator response to nitroglycerin was also used as the baseline nitroglycerin-induced vasodilatation before cigarette smoking when the acute effects of cigarette smoking on the nitroglycerin-induced vasodilatation were analyzed in 15 smokers.

Biochemical Assays

Vitamin C. The serum levels of vitamin C were determined by high-performance liquid chromatography (27). Blood sampling or measurement of vitamin C was obtained from all study subjects before vitamin C infusion on the first study day. The time course of changes in serum levels of vitamin C was examined over a period of 30 min during intravenous infusion of vitamin C at a constant dose of 10 mg/min in seven nonsmokers (Fig. 1).

TBARS. The content of lipid peroxide in plasma was determined using TBARS as a marker (4). Briefly, 2.0 ml of trichloroacetic acid-thiobarbituric acid (TBA)-HCl reagent was added to 1.0 ml of sample and vortexed. To minimize peroxidation during the assay procedure, butylated hydroxytoluene was added to the TBA reagent mixture. The results were expressed as malondialdehyde (MDA) equivalent content (nanomoles of MDA per milliliter of plasma).

Statistics Analysis

Data are expressed as means ± SD. Comparisons between groups were performed by two-tailed unpaired t-test for
continuous variables or χ² test for categorical variables. Comparisons between the infusions of saline and saline plus vitamin C were performed by paired t-test for continuous variables. Comparisons between ‘before’ and ‘after’ cigarette smoking were also performed by paired t-test for continuous variables. Serial changes in serum levels of vitamin C after vitamin infusion were compared using repeated-measures analysis of variance and a post hoc Bonferroni test. Correlations between serum levels of vitamin C and flow-dependent vasodilation were examined using linear regression analysis. Statistical significance was defined as P < 0.05.

RESULTS

Hemodynamic Variables

There were no significant differences in the baseline values of heart rate, blood pressure, brachial arterial diameter, or brachial arterial blood flow between the smokers and nonsmokers. Heart rate, blood pressure, brachial arterial diameter, and brachial arterial blood flow were not affected by vitamin C administration in either group (Table 2).

There were no significant differences in systolic blood pressure, brachial arterial diameter, or brachial arterial blood flow before and 10 min after cigarette smoking. However, heart rate, diastolic blood pressure, and mean blood pressure were higher after cigarette smoking (Table 3).

Flow-Dependent Vasodilation

Flow-dependent vasodilation was reduced in smokers compared with that in nonsmokers (5.3 ± 1.9 vs. 9.2 ± 1.5%, P < 0.0001) (Fig. 2). Vitamin C administration improved the flow-dependent vasodilation in smokers (5.3 ± 1.9 to 9.0 ± 3.2%, P < 0.001) but not in nonsmokers (9.2 ± 1.5 to 9.5 ± 2.1%, P = not significant (NS)) (Fig. 2). Vitamin C administration did not affect the increases in either the brachial blood flow during reactive hyperemia or the brachial diameter after nitroglycerin administration in either group (Table 2).

<table>
<thead>
<tr>
<th>Table 2. Effects of vitamin C administration on hemodynamic variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smokers</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
</tr>
<tr>
<td>Mean blood pressure, mmHg</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
</tr>
<tr>
<td>Resting arterial diameter, mm</td>
</tr>
<tr>
<td>Increase in arterial blood flow, %</td>
</tr>
<tr>
<td>Increase in diameter after nitroglycerin administration, %</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD for 20 smokers and 20 nonsmokers. Measurements of flow-dependent vasodilation were performed after infusion of saline or saline + vitamin C.

Cigarette smoking acutely worsened the impairment of flow-dependent vasodilation in smokers (5.4 ± 1.8 to 1.5 ± 1.3%, P < 0.01) (Fig. 3). However, vitamin C administration prevented the acute impairment of flow-dependent vasodilation by cigarette smoking (8.9 ± 2.2 to 7.3 ± 2.4%, P = NS) (Fig. 3).

<table>
<thead>
<tr>
<th>Table 3. Effects of cigarette smoking on hemodynamic variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Smoking</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
</tr>
<tr>
<td>Mean blood pressure, mmHg</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
</tr>
<tr>
<td>Resting arterial diameter, mm</td>
</tr>
<tr>
<td>Resting arterial blood flow, ml/min</td>
</tr>
<tr>
<td>Increase in arterial blood flow, %</td>
</tr>
<tr>
<td>Increase in diameter after nitroglycerin administration, %</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD. Measurements of flow-dependent vasodilation were performed before and 10 min after cigarette smoking. NS, not significant.
not affect the increases in either the brachial blood flow during reactive hyperemia or the brachial diameter after nitroglycerin administration (Table 3).

Levels of Serum Vitamin C and Plasma TBARS

Serum levels of vitamin C at baseline were lower in smokers than in nonsmokers (36.8 ± 12.1 vs. 56.3 ± 11.2 μmol/l, P < 0.001). Plasma levels of TBARS at baseline were higher in smokers than in nonsmokers (1.8 ± 0.4 vs. 1.3 ± 0.3 nmol/ml, P < 0.05) (Fig. 4). The infusion of vitamin C (10 mg/min for 20 min) significantly increased serum vitamin C levels when the flow-dependent vasodilation was examined (50.2 ± 9.3 to 168 ± 35.1 μmol/l, P < 0.01) (Fig. 1). Vitamin C administration decreased the plasma levels of TBARS in smokers (1.8 ± 0.4 to 1.3 ± 0.3 nmol/ml, P < 0.05)
but did not affect the levels in nonsmokers (1.3 ± 0.3 to 1.2 ± 0.3 nmol/ml, P = NS) (Fig. 4).

Cigarette smoking acutely increased the plasma TBARS levels (1.7 ± 0.4 to 2.2 ± 0.5 nmol/ml, P < 0.05) (Fig. 5). However, vitamin C administration prevented the acute increase in plasma TBARS levels by cigarette smoking (1.3 ± 0.5 to 1.5 ± 0.5 nmol/ml, P = NS) (Fig. 5).

**Correlation Between Serum Level of Vitamin C Versus Flow-Dependent Vasodilation**

There was a positive correlation between serum levels of vitamin C versus flow-dependent vasodilation after saline infusion on the first study day in both the smokers and nonsmokers (r = 0.597, P < 0.0001) (Fig. 6).

**DISCUSSION**

The present study showed that the percent increase in the arterial diameter during reactive hyperemia was smaller in smokers than in nonsmokers. However, there was no significant difference in the percent increase in arterial blood flow during reactive hyperemia between the smokers and nonsmokers. Because the response to nitroglycerin, a direct relaxant of smooth muscle, was preserved in both groups, the flow-mediated, endothelium-dependent vasodilation of the brachial arteries is thought to be impaired in the smokers compared with that in the nonsmokers. The serum levels of vitamin C were decreased and the plasma levels of TBARS were increased in chronic smokers compared with these levels in nonsmokers. Vitamin C administration improved the impairment of endothelium-dependent vasodilation in smokers in association with a decrease in the plasma TBARS, whereas it had no effects in nonsmokers. These findings suggest that the increased oxidative stress contributes to endothelial dysfunction in chronic smokers. Several reports (9, 14) have shown that antioxidants, especially vitamin C, improved the impaired endothelium-dependent vasodilation in chronic smokers or in patients with coronary artery disease. However, they did not examine the effects of vitamin C on oxidative stress. The present study first showed that vitamin C infusion improved endothelium-dependent vasodilation in association with a reduction in TBARS, an indicator of oxidative stress.

The precise mechanism of the impairment of endothelium-dependent vasodilator responses in chronic smokers still remains undetermined in the present study, although oxidative stress may be suggested to play a role in the mechanism. Cigarette smoke has been reported (3, 9) to contain nicotine and large amounts of free radicals, such as superoxide anion and hydroxyl radicals. Our group (18) has shown that superoxide anions in cigarette smoke degrade endothelium-derived nitric oxide, leading to a decrease in flow-dependent vasodilation. Our group (13) has recently demonstrated that nitric oxide bioactivity in coronary circulation was decreased in long-term smokers. Furthermore, superoxide anions and nitric oxide can combine to form peroxynitrite, a highly reactive intermediate with cytotoxic potency (21). Thus free radicals in cigarette smoke may not only degrade nitric oxide released from endothelium but also produce highly reactive intermediates, resulting in endothelial injury. In this study, cigarette smoking produced acute impairment of the flow-dependent vasodilation in association with an increase in TBARS. Furthermore, the suppressive effects of cigarette smoking on endothelium-dependent vasodilation were attenuated by coinjection of the antioxidant vitamin C. These findings suggest

![Graph showing correlation between serum level of vitamin C and arterial diameter increase](image-url)
that cigarette smoking may increase oxidative stress, leading to acute impairment of endothelium-dependent vasodilation.

Several basic experiments (2, 23) have shown that cigarette smoke is capable of inducing endothelial toxicity. The endothelial dysfunction by long-term smoking has been shown to be partially caused by a direct toxic effect on human endothelial cells. In the present study, endothelium-dependent vasodilation was improved by the antioxidant vitamin C and worsened by cigarette smoking. These findings suggest that the impairment of endothelial function in chronic smokers may be at least partially reversible, in agreement with a previous report (5).

Study Limitation

The principal finding of the present study was that vitamin C improved flow-dependent vasodilation of the brachial artery in smokers but not in nonsmokers. However, any treatment could possibly improve the brachial responses somewhat by a nonspecific effect of infusion, because smokers had a profound impairment in flow-dependent vasodilation before vitamin C infusion. Therefore, when we examined the flow-dependent vasodilation without vitamin C infusion, we infused the same volume of saline in the same manner as the vitamin C infusion and examined the effects of saline as a placebo in the flow-dependent vasodilation. Thus the beneficial effects of vitamin C in smokers is unlikely to be nonspecific.

An increased concentration of end products of lipid peroxidation is the evidence most frequently quoted for the involvement of free radicals in human disease. Many assays are available to measure lipid peroxidation, but no single assay reflects the whole process accurately. The measurement of TBARS is susceptible to artifacts caused by variations in sample lipid content and in iron contamination of the reagents (8). In this study, we prevented amplification of peroxidation during the assay by adding the chain-breaking antioxidant butylated hydroxytoluene to the samples before adding the TBA reagents.

Clinical Implications

Cigarette smoking, which was shown to impair the endothelium-dependent vasodilation in the present and previous studies (5, 9, 13), is a significant risk factor for coronary artery disease. The impairment of endothelial function in smokers is at least partially reversible, as we showed in the present study, and it is possible that the impaired endothelial function in smokers may be restored by cessation of smoking. Indeed, a previous study showed that risk for myocardial infarction is reduced by 50% within 1 yr of cessation of smoking (30).

In conclusion, endothelium-dependent vasodilation in the brachial artery is impaired in chronic smokers, and this impairment is improved by vitamin C infusion in association with a decrease in TBARS. Furthermore, cigarette smoking produces acute impairment of flow-dependent vasodilation in smokers in association with an increase in TBARS. These findings suggest that the increased oxidative stress may contribute to the endothelial dysfunction in smokers.

This study was supported in part by Grant-in-Aid for Scientific Research A-08407019 from the Ministry of Education, Science, Sports and Culture in Japan and the Smoking Research Foundation Grant for Biomedical Research, Tokyo, Japan.

Address for reprint requests: H. Yasue, Div. of Cardiology, Kuma- moto Univ. School of Medicine, H ornamento City 860, Japan.

Received 21 March 1997; accepted in final form 17 June 1997.

REFERENCES


16. Moreyra, A. E., C. R. Lacy, A. C. Wilson, A. Kumar, and J. B. Kostis. Arterial blood nicotine concentration and coronary vaso-


