Endothelial Dysfunction, Oxidative Stress, and Risk of Cardiovascular Events in Patients With Coronary Artery Disease

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Background—Endothelial function is impaired in coronary artery disease and may contribute to its clinical manifestations. Increased oxidative stress has been linked to impaired endothelial function in atherosclerosis and may play a role in the pathogenesis of cardiovascular events. This study was designed to determine whether endothelial dysfunction and vascular oxidative stress have prognostic impact on cardiovascular event rates in patients with coronary artery disease.

Methods and Results—Endothelium-dependent and -independent vasodilation was determined in 281 patients with documented coronary artery disease by measuring forearm blood flow responses to acetylcholine and sodium nitroprusside using venous occlusion plethysmography. The effect of the coadministration of vitamin C (24 mg/min) was assessed in a subgroup of 179 patients. Cardiovascular events, including death from cardiovascular causes, myocardial infarction, ischemic stroke, coronary angioplasty, and coronary or peripheral bypass operation, were studied during a mean follow-up period of 4.5 years. Patients experiencing cardiovascular events (n=91) had lower vasodilator responses to acetylcholine ($P<0.001$) and sodium nitroprusside ($P<0.05$), but greater benefit from vitamin C ($P<0.01$). The Cox proportional regression analysis for conventional risk factors demonstrated that blunted acetylcholine-induced vasodilation ($P=0.001$), the effect of vitamin C ($P=0.001$), and age ($P=0.016$) remained independent predictors of cardiovascular events.

Conclusions—Endothelial dysfunction and increased vascular oxidative stress predict the risk of cardiovascular events in patients with coronary artery disease. These data support the concept that oxidative stress may contribute not only to endothelial dysfunction but also to coronary artery disease activity. **(*Circulation*. 2001;104:2673-2678.)**

Key Words: antioxidants ■ coronary disease ■ endothelium ■ free radicals ■ prognosis

The endothelium plays an integral role in the regulation of vascular tone, platelet activity, leukocyte adhesion, and thrombosis and is intimately involved in the development of atherosclerosis. Endothelial dysfunction has been observed in patients with established coronary artery disease or coronary risk factors, both in the coronary and peripheral vasculature.[1] Therapeutic interventions with lipid-lowering drugs,[2–4] ACE inhibitors,[5] physical activity,[6,7] and antioxidant agents[3,8–10] have been shown to improve endothelial function in coronary and peripheral vessels. This systemic manifestation and improvement of endothelial function suggests that a common mechanism may contribute to endothelial dysfunction in the coronary and peripheral circulation. An increasing body of evidence suggests that increased oxidative stress accounts for a significant proportion of endothelial dysfunction. Increased production of oxygen-derived free radicals such as the superoxide anion has been linked to impaired endothelial vasomotor function in experimental models of atherosclerosis.[11–13] Accordingly, treatment with antioxidants has been shown to improve coronary and peripheral endothelial function in patients with coronary artery disease or coronary risk factors.[3,8–10,14]

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Less information is available about the prognostic impact of endothelial dysfunction and oxidative stress on atherosclerotic disease progression and cardiovascular risk. Very recent observations showed that depressed coronary vasoreactivity is associated with increased cardiovascular event rates.[15,16] However, the mechanism(s) underlying this association between endothelial dysfunction and cardiovascular risk have not been defined.

Epidemiological studies indicate an association between increased intake of dietary antioxidants and reduced risk of coronary events.[17–19] Thus, the hypothesis has been forwarded that an imbalance between increased oxidative stress and impaired antioxidant defense may not only affect endothelial function, but may also contribute to atherosclerotic disease progression. Therefore, we prospectively investigated whether systemic endothelial dysfunction and vascular oxidative stress, as assessed by the response to the antioxidant vitamin C, have prognostic impact on adverse long-term outcomes in patients with coronary artery disease.

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Methods

Patient Population

Between January 1994 and October 1998, 281 consecutive patients who had been referred for assessment of coronary artery disease were studied prospectively. The inclusion criteria were angiographically documented coronary artery disease and a stable condition. Each subject was screened by a complete history, physical examination, and laboratory analysis. Exclusion criteria included unstable angina, recent myocardial infarction (<5 months), recent coronary angioplasty (<5 months), valvular heart disease, evidence of heart failure, uncontrolled hypertension, and/or significant endocrine, hepatic, renal, or inflammatory disease. Vasoactive medications, including calcium-channel blockers, ACE inhibitors, and long-acting nitrates, were withheld for ≥24 hours before the study. Patients were taking aspirin 100 mg/d on a long-term basis, with the exception of 11 patients who were taking clopidogrel. All female subjects were postmenopausal. Only 5 women were receiving hormone replacement therapy. Risk factors were assessed at the time of the endothelial function test. No caffeine intake or smoking was allowed before the study. The study was approved by the local ethics committee, and informed consent was obtained from all participants.

Assessment of Vascular Function

All studies were performed after a 12-hour overnight fast with the subjects lying supine in a quiet, temperature-controlled room (22°C to 24°C). Using sterile conditions and 2% lidocaine, a 20-gauge polyethylene catheter was inserted into the brachial artery of the nondominant arm to measure blood pressure and drug infusion. Forearm blood flow (FBF) was measured by venous occlusion plethysmography with calibrated mercury-in-silastic strain gauges, as previously described.[10] During FBF measurement, circulation to the hand was excluded by a wrist cuff that was inflated to 40 mm Hg above systolic blood pressure. At the beginning of each study protocol, normal saline (0.9% sodium chloride) was infused intra-arterially at a rate of 0.4 mL/min. Endothelium-dependent vasodilation was assessed by infusing acetylcholine (ACh) in increasing concentrations of 7.5, 15, and 30 μg/min (ACh1 to 3) into the brachial artery. Sodium nitroprusside (SNP) was infused to assess endothelium-independent vasodilation (1, 3, and 10 μg/min). The sequence of ACh and SNP infusion was randomized. In a subgroup of 179 patients, the dose-response curve to ACh was repeated during coinfusion of vitamin C at 24 mg/min. This dose of vitamin C was chosen to provide a final plasma concentration of ≈10 mmol/L.[8,20] Vitamin C infusion was started 10 minutes before ACh infusion and was continued throughout. Finally, the dose-response curve to SNP was repeated during coinfusion with vitamin C. A 30-minute washout was allowed between each dose-response curve.

Long-Term Follow-Up

Follow-up data were obtained using questionnaires sent to patients and/or primary physicians. In addition, hospital records were reviewed. During long-term follow-up, the following events were assessed. Death from cardiovascular causes was defined as death due to a myocardial or cerebral infarction or documented sudden cardiac death. Myocardial infarction, ischemic stroke, PTCA, CABG, and peripheral bypass revascularization were confirmed by reviewing hospital records. Medical therapy, especially with ACE inhibitors, lipid-lowering drugs, and antioxidants, was documented.

Statistical Analysis

Data of baseline characteristics and cardiovascular events at follow-up are expressed as mean±SD or n (%). Responses to ACh and SNP, with and without vitamin C, are presented as mean±SEM and were analyzed by ANOVA for repeated measures. Scheffe's test was applied for multiple comparison testing.

Cumulative event rates were estimated by Kaplan-Meier survival curves, and probability values were determined with the log-rank test. For Kaplan-Meier analyses, ACh- and SNP-induced vasodilation and the effect of vitamin C were divided into values below and above the median. Cox proportional hazards regression analysis was performed to determine independent predictors of cardiovascular events for all patients studied. This Cox model included the following variables: age; sex; the presence of hypertension; smoking; diabetes; previous infarction; serum levels of total cholesterol, LDL cholesterol, and HDL cholesterol; extent of vessel disease; and FBF responses to ACh and SNP. An additional Cox regression analysis was performed for the subgroup of patients who also received vitamin C. $P<0.05$ was considered statistically significant.

TABLE 1. Patient Characteristics

	All Patients (n=276)	Patient With Events (n=91)	Patients Without Events (n=185)
Age, y	60±8	63±7	59±9
Men, n (%)	221 (80)	73 (80)	148 (80)
Women/HRT, n	55/5	18/2	37/3
Hypertension, n (%)	106 (38)	36 (40)	70 (38)
Smokers, n (%)	91 (33)	32 (35)	59 (32)
Diabetes mellitus, n (%)	57 (21)	20 (22)	37 (20)
Total cholesterol, mg/dL	232±43	224±44	226±42
LDL cholesterol, mg/dL	140±36	140±38	139±35
HDL cholesterol, mg/dL	45±10	44±10	45±10
Triglycerides, mg/dL	157±14	161±16	156±13
Extent of disease, n (%)			
1 Vessel	102 (37)	35 (38)	67 (36)
2 Vessels	91 (33)	30 (33)	61 (33)
3 Vessels	83 (30)	26 (29)	57 (31)
Previous infarction, n (%)	104 (38)	36 (40)	68 (37)

Data are presented as mean±SD or n (%). HRT indicates hormone replacement therapy.

Results

Patient Characteristics

We included 281 patients in the study. Of these, 5 patients were excluded because follow-up could not be done. The baseline characteristics of the remaining 276 patients are summarized in Table 1. Patients were followed for a mean of 53 months (range, 30 to 87 months). At follow-up, we documented 120 cardiovascular events in 91 patients (Table 2). Sixty-nine patients experienced 1 event, 15 patients had 2 events, and 7 patients had 3 events. In addition, 3 patients died of cancer.

On univariate analysis, only age ($P=0.01$) and diabetes ($P=0.03$) were significantly associated with the occurrence

TABLE 2. Cardiovascular Events in the Study Population (n=276) at Follow-Up

Event	n (%)
Death from cardiovascular causes	18 (6.5)
Myocardial infarction	25 (9.1)
Ischemic stroke	6 (2.2)
PTCA	46 (16.7)
CABG	19 (6.9)
Peripheral bypass revascularization	6 (2.2)

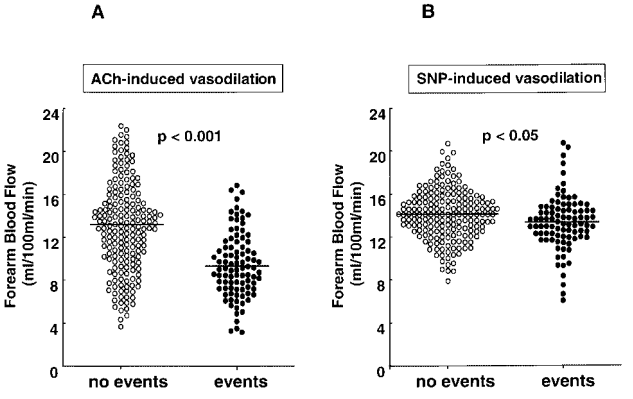

Figure 1. Maximal FBF responses to ACh 30 μg/min (A) and SNP 10 μg/min (B) in patients with (filled circles, n=91) and without (open circles, n=185) cardiovascular events during long-term follow-up.

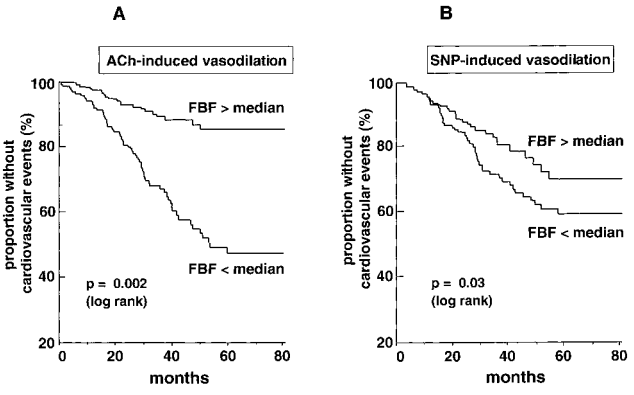

Figure 2. Cumulative proportion of patients without cardiovascular events during follow-up (Kaplan-Meier). Maximal ACh-induced vasodilation (A) and SNP-induced vasodilation (B) are divided into values below and above the median.

of cardiovascular events. The use of ACE inhibitors, lipid-lowering agents, and antioxidants was comparable between groups with and without cardiovascular events.

Peripheral Vascular Function and Prognosis

Intra-arterial infusion of ACh increased FBF in the total group of patients from a basal FBF of 2.7±0.1 mL/min per 100 mL to 5.8±0.1 (ACh1), 8.3±0.2 (ACh2), and (maximally) 11.7±0.3 mL/min per 100 mL of tissue (ACh3). Patients experiencing cardiovascular events during follow-up had a significantly lower vasodilator response compared with patients without events ($P<0.001$ by ANOVA). In patients with events, ACh1-induced FBF was 4.7±0.2 mL/min per 100 mL (6.1±0.2 mL/min per 100 mL in patients without events) and ACh2-induced FBF was 6.5±0.3 mL/min per 100 mL (9.1±0.3 mL/min per 100 mL in patients without events). The FBF responses to ACh3 are illustrated in Figure 1A. There were significant differences between patients with and without cardiovascular events.

The intra-arterial infusion of SNP increased FBF in the total group from 2.7±0.1 mL/min per 100 mL to 5.7±0.2 (SNP1), 9.5±0.2 (SNP2), and 14.2±0.2 mL/min per 100 mL (SNP3). The vasodilator response to SNP was slightly but significantly reduced in patients with cardiovascular events compared with patients free of events ($P<0.05$ by ANOVA). In patients with events, SNP1-induced FBF was 5.6±0.2 mL/min per 100 mL (5.9±0.3 mL/min per 100 mL in patients without events), and SNP2-induced FBF was 9.2±0.4 mL/min per 100 mL (9.9±0.3 mL/min per 100 mL in patients without events). The FBF responses to SNP3 are illustrated in Figure 1B. Again, these responses showed slightly, but significantly, reduced FBF responses in patients with events.

The cumulative proportion of patients with cardiovascular events according to the vasodilator response to ACh and SNP is shown in Figure 2. The incidence of cardiovascular events increased significantly with decreasing vasodilator response to ACh (Figure 2A). There was a small but significant increase in cardiovascular events with decreasing vasodilator response to SNP (Figure 2B).

Effect of Vitamin C and Prognosis

A subgroup of 179 patients received an intra-arterial infusion of vitamin C and was followed for a mean of 45 months (range, 31 to 74 months). At follow-up, 67 cardiovascular events occurred in 49 patients. The vasodilator response to ACh was significantly lower in patients experiencing cardiovascular events compared with patients without events (Figure 3A). Coinfusion of vitamin C improved FBF responses to ACh in patients with and without events. However, the effect of vitamin C was significantly larger in patients experiencing

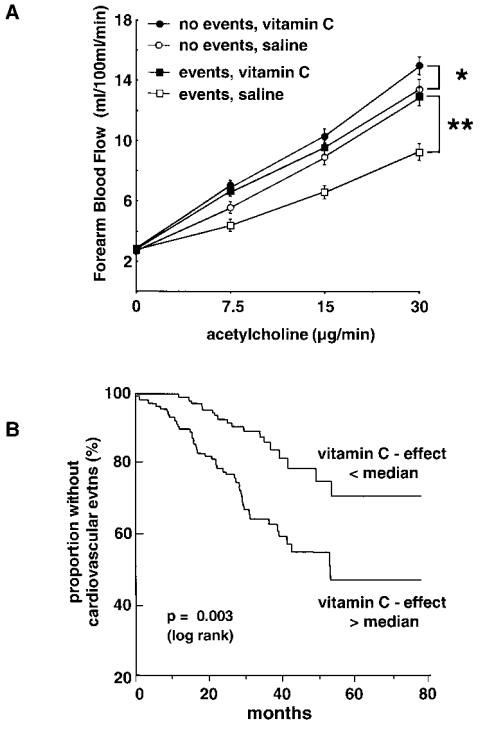

Figure 3. A, ACh-induced vasodilation in patients with (squares) and without (circles) cardiovascular events during saline and vitamin C infusion. Vitamin C improved ACh-induced vasodilation in both groups. However, the effect of vitamin C was significantly larger in patients with events (n=49) compared with patients without events (n=130). *$P<0.05$ vs saline; **$P<0.001$ vs saline (ANOVA). B, Kaplan-Meier analysis demonstrating cumulative proportion of patients without cardiovascular events during follow-up. Effect of vitamin C on ACh-induced vasodilation (30 μg/min) is divided into values below and above the median.

TABLE 3. Cox Regression Analyses (n=276)

Variable	OR	95% CI	P
Age	1.05	1.02–1.09	0.002
Sex	1.76	0.83–3.76	0.14
Hypertension	1.13	0.67–2.92	0.64
Smoking	0.66	0.38–1.16	0.15
Diabetes mellitus	0.54	0.26–1.11	0.09
Total cholesterol	0.99	0.98–1.01	0.27
LDL cholesterol	1.01	0.99–1.02	0.15
HDL cholesterol	0.99	0.96–1.02	0.45
Extent of vessel disease	1.34	0.46–4.49	0.45
Previous infarction	1.43	0.62–2.86	0.67
SNP-induced vasodilation	0.88	0.73–1.07	0.19
ACh-induced vasodilation	0.84	0.79–0.91	<0.001

OR indicates odds ratio; CI, confidence interval.

cardiovascular events during follow-up compared with patients without events ($P<0.01$ by ANOVA). Using Kaplan-Meier analyses, the incidence of cardiovascular events increased significantly with increasing effect of vitamin C on ACh-induced FBF (Figure 3B). Infusion of vitamin C had no significant effect on SNP-induced vasodilation in patients with and without events.

Cox Regression Analysis

Cox proportional hazards regression was used to analyze follow-up data and to determine independent predictors of cardiovascular events. Analysis for all patients (n=276) included the following variables: age, sex, the presence of hypertension, smoking, diabetes, previous infarction, serum levels of cholesterol (total, LDL, and HDL), extent of vessel disease, and FBF responses to ACh and SNP. In this analysis, ACh-induced vasodilation ($P<0.001$) and age ($P=0.002$) were significantly associated with the occurrence of cardiovascular events. Diabetes ($P=0.09$) and smoking ($P=0.15$) did not reach significance (Table 3). In addition, Cox regression analysis for the subgroup of patients investigated with vitamin C (n=179) was performed. When the vitamin C effect on ACh-response was included into the regression analysis, the remaining independent predictors of cardiovascular events were ACh-induced vasodilation ($P=0.001$), effect of vitamin C on ACh-response ($P=0.001$), and age ($P=0.016$) (Table 4).

Discussion

Endothelium-dependent vasodilation has been shown to be impaired in patients with atherosclerosis or risk factors for atherosclerosis. Numerous studies have demonstrated that this impairment occurs in both the coronary and peripheral circulation.[8,9,21,22] Although the correlation between the coronary and peripheral circulation is reported to be only modest,[21,23] common underlying mechanism(s) seem to account for the observed depression of endothelial function. This suggestion of a systemic nature of endothelial dysfunction is further supported by the observation that different therapeutic interventions such as lipid-lowering,[2,4] ACE inhibition,[5] physical activit,y[6,7] and antioxidant therapy[3,8–10] exert their beneficial effects on both the coronary and peripheral circulation.

In experimental animal models of atherosclerosis, hypercholesterolemia, hypertension, and diabetes, associations between oxidative stress and impaired endothelial function have been demonstrated.[11–13] Among many biological changes that occur in the vessel wall under these conditions, reduced bioavailability of nitric oxide (NO) in a setting of increased superoxide anion levels seems to be a uniform underlying abnormality. Recent studies extended this potential mechanism to patients with coronary artery disease by demonstrating increased superoxide production of human blood vessels in association with endothelial vasomotor dysfunction and with clinical risk factors.[24,25] Furthermore, endothelial dysfunction in patients with coronary artery disease or coronary risk factors could be reversed by the administration of agents capable of scavenging superoxide, such as vitamin C.[8–10,20] These findings suggest that increased oxidative stress may be an important mechanism for impaired endothelial function in patients with atherosclerosis or cardiovascular risk factors.

There is a paucity of information regarding the role of endothelial dysfunction on the progression of atherosclerotic disease and the cardiovascular event rate. Very recent studies indicate that abnormal coronary vasoreactivity in patients with coronary artery disease is a predictor of increased cardiovascular risk.[15,16] The present study extends these observations to the systemic circulation of patients with coronary disease. Our data indicate that peripheral endothelial vasodilator dysfunction is associated with increased cardiovascular risk and may serve as an independent predictor of atherosclerotic disease activity. These findings are in line with a recent investigation demonstrating the prognostic value of endothelium-dependent vasodilation in the brachial artery.[26] Patients in these studies had only mild coronary artery disease, and therefore the event rate was low.[26] We included patients with well-established coronary vessel disease and found an event rate comparable to those of other follow-up studies in patients with CAD.[27]

The mechanisms responsible for the association between impaired endothelial function and the progression of atherosclerotic disease are unknown. After finding a similar relationship between abnormal coronary vasodilator response and the long-term outcome of coronary heart disease, Schachinger et al[16] speculated that a blunted dilator response mirrors the oxidative stress imposed on the vascular wall, which in turn will determine disease activity. In fact, the present study underscores this potential mechanism of oxidative stress–driven progression of atherosclerotic disease, because the beneficial effect of vitamin C was significantly increased in

TABLE 4. Independent Predictors of Cardiovascular Events

Variable	OR	95% CI	P
Age	1.04	1.01–1.08	0.016
ACh-induced vasodilation	0.90	0.85–0.96	0.001
Vitamin C effect on ACh-induced vasodilation*	1.17	1.07–1.27	0.001

OR indicates odds ratio; CI, confidence interval.
*Only 179 patients were treated with vitamin C.

patients who experienced cardiovascular events. The oxygen-derived free radical superoxide combines with NO in a diffusion-limited reaction ($k=6.7\times10^{10}$ mol/L per s) that is ≈3 times faster than the dismutation of superoxide by superoxide dismutase. This reaction produces peroxynitrite, a compound with limited NO-like bioactivity, thereby "shunting" NO away from its typical targets, such as vasodilation and inhibition of platelets. This latter effect may be important for triggering vascular events because impaired NO bioactivity is predictive of atherosclerotic disease activity.[28]

Vitamin C is an extremely effective antioxidant, and it is an efficient scavenger of many reactive oxygen species, including superoxide anions[29] and peroxynitrite. We and others have demonstrated that the beneficial effect of vitamin C at 24 mg/min on endothelial dysfunction in patients with risk factors or coronary artery disease is specific, because it was observed neither in healthy control subjects nor on the endothelium-independent vasodilation induced by SNP or nitroglycerin.[8–10] Thus, the results of these studies are consistent with the concept that the scavenging of increased oxygen-derived free radicals may account for the beneficial effects of vitamin C in these settings. The salient finding of the present study is that the positive response to vitamin C was an independent predictor of a higher risk of cardiovascular events. This observation supports the concept that oxidative stress contributes to the progression of atherosclerotic disease and may, therefore, be an important determinant of clinical events.

Given the conflicting data from clinical trials of antioxidants in cardiovascular disease, the emergence of such a clear result may seem quite a surprise. As mentioned above, however, it is a quite consistent finding that vitamin C can improve endothelial dysfunction in diseases where oxidative stress may play a role, such as hypercholesterolemia, long-term smoking, congestive heart failure, hypertension, and diabetes mellitus. Therefore, we think that vitamin C's effects on ACh-induced vasodilation can be used as a surrogate parameter reflecting oxidative stress in vascular tissue. On the basis of the very low rate constant of the reaction between vitamin C and superoxide ($k=3\times10^{5}$ mol/L per s), vitamin C must be given in very high concentrations to compete successfully with NO for superoxide anion. Accordingly, high concentrations of vitamin C have to be given intra-arterially in these protocols. Although we can improve endothelial dysfunction with this approach in the short-term, it may also be easy to understand why therapies with oral vitamin C have been shown to be rather unsuccessful.

In the present study, we also found that endothelium-independent vasodilation to SNP was mildly impaired in patients experiencing cardiovascular events compared with patients without events. Interestingly, in the setting of hypercholesterolemia, superoxide has also been shown to be increased in endothelial cells and in smooth muscle cells,[28] which in turn may inhibit the activity of the NO target enzyme, soluble guanylyl cyclase.[30] In addition, a recent investigation demonstrated that vascular oxidative stress is associated with a reversible impairment of activity of the cGMP-dependent protein kinase, leading to reduced NO/cGMP-dependent relaxation.[31]

Noteworthy is the growing evidence for a common pathway mediating oxidative stress, vascular dysfunction, and inflammatory gene induction.[32] Recent studies demonstrated that a systemic inflammatory response leads to an impairment of endothelial function in both resistance and conduit vessels.[33] Furthermore, blunted systemic endothelial vasoreactivity was found to be related to elevated plasma levels of C-reactive protein in patients with coronary artery disease.[34] On the basis of these observations, we conclude that changes in endothelial activity may emphasize the link between inflammation and the risk of cardiovascular events.

In conclusion, the present study demonstrates that blunted systemic endothelial vasoreactivity represents an independent predictor for increased cardiovascular risk. Although no measures of endogenous antioxidant defense or of oxidant stress in plasma were performed, we think that increased vascular oxidative stress, as indicated by the higher response to vitamin C, may represent an important underlying mechanism for endothelial dysfunction and for the pathogenesis of cardiovascular events.

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