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Long-Term Ascorbic Acid Administration Reverses Endothelial Vasomotor Dysfunction in Patients With Coronary Artery Disease

Noyan Gokce, MD; John F. Keaney, Jr, MD; Balz Frei, PhD; Monika Holbrook, MS; Mariusz Olesiak, BS; Benoy J. Zachariah, MD; Christiana Leeuwenburgh, PhD; Jay W. Heinecke, MD; Joseph A. Vita, MD

Background—Loss of endothelium-derived nitric oxide (EDNO) contributes to the clinical expression of coronary artery disease (CAD). Increased oxidative stress has been linked to impaired endothelial vasomotor function in atherosclerosis, and recent studies demonstrated that short-term ascorbic acid treatment improves endothelial function.

Methods and Results—In a randomized, double-blind, placebo-controlled study, we examined the effects of single-dose (2 g PO) and long-term (500 mg/d) ascorbic acid treatment on EDNO-dependent flow-mediated dilation of the brachial artery in patients with angiographically established CAD. Flow-mediated dilation was examined by high-resolution vascular ultrasound at baseline, 2 hours after the single dose, and 30 days after long-term treatment in 46 patients with CAD. Flow-mediated dilation improved from 6.6 ± 3.5% to 10.1 ± 5.2% after single-dose treatment, and the effect was sustained after long-term treatment (9.0 ± 3.7%), whereas flow-mediated dilation was 8.6 ± 4.7% at baseline and remained unchanged after single-dose (7.8 ± 4.4%) and long-term (7.9 ± 4.5%) treatment with placebo (P < 0.005 by repeated-measures ANOVA). Plasma ascorbic acid concentrations increased from 41.4 ± 12.9 to 115.9 ± 34.2 μmol/L after single-dose treatment and to 95.0 ± 36.1 μmol/L after long-term treatment (P < 0.001).

Conclusions—In patients with CAD, long-term ascorbic acid treatment has a sustained beneficial effect on EDNO action. Because endothelial dysfunction may contribute to the pathogenesis of cardiovascular events, this study indicates that ascorbic acid treatment may benefit patients with CAD. (Circulation. 1999;99:3234-3240.)

Key Words: coronary disease ■ endothelium ■ vitamins

Endothelium-derived nitric oxide (EDNO) plays a critical role in the regulation of vasomotor tone, platelet activity, and leukocyte adhesion to the vascular wall.1 EDNO action is impaired in coronary artery disease (CAD) and may contribute to its clinical expression.2 Recent studies suggest that increased vascular production of superoxide anion contributes to impaired EDNO action in atherosclerosis.3 Another feature of atherosclerosis is vascular accumulation of oxidized LDL (ox-LDL), which is cytotoxic, inhibits NO release from endothelial cells, and may inactivate NO directly.4

A link between these abnormalities and impaired EDNO action is supported by studies demonstrating improved endothelium-dependent dilation after treatment with antioxidants, including ascorbic acid (vitamin C).5-9 Ascorbic acid scavenges superoxide anion,10 inhibits LDL oxidation,11 and also plays an important role in the control of intracellular redox state.12 We previously demonstrated that a single oral dose of ascorbic acid improves brachial artery flow-mediated dilation in patients with CAD.5 Other investigators have also reported that short-term ascorbic acid treatment significantly improves EDNO-mediated vasodilation in forearm resistance vessels of patients with coronary risk factors6,8,9 and the coronary arteries of patients with hypertension.2

Although the short-term effects of ascorbic acid on endothelial function are well established, the potential role of ascorbic acid for long-term therapy for CAD has not previously been examined. The purpose of this study was to determine whether long-term ascorbic acid supplementation is associated with sustained improvement in endothelial function and to gain insights into the mechanisms of that effect.

Methods

Research Subjects

Patients with angiographically documented CAD (≥1 stenosis >50%) were eligible for study. Patients were excluded if they had unstable angina, acute myocardial infarction within 1 week, uncontrolled hypertension, congestive heart failure, or any other condition...
that would preclude safely withholding vasoactive medications. Other exclusion criteria included clinically significant valvular heart disease, use of antioxidant vitamins (vitamin C or E or β-carotene) within 1 month, initiation of cholesterol-lowering therapy within 6 months, and estrogen replacement therapy. All subjects provided written informed consent.

### Study Protocol
Each subject made 2 visits 30 days apart. Subjects were instructed not to eat or drink on the morning of each visit and, if applicable, not to smoke for at least 24 hours. All medications were held for >12 hours, and all long-acting vasoactive medications were held for >24 hours before evaluation.

During the first visit, vital signs were recorded and fasting blood and urine samples were obtained. Flow-mediated dilation of the brachial artery was determined noninvasively with vascular ultrasound as previously described.5,13 Hyperemia was induced by a cuff brachial artery was determined noninvasively with vascular ultrasound.

Ultrasound images were obtained at baseline with ascorbic acid (2 g PO) or matched placebo tablets (Leiner Health Products). Two hours after treatment, vital signs were recorded, a blood sample was obtained, and the noninvasive assessment of flow-mediated dilation and nitroglycerin-mediated dilation were again performed. Ultrasound images were digitized, and vessel diameter was measured by use of validated, customized software by personnel blinded to both image sequence and treatment assignment.5 Maximal hyperemic flow was estimated by use of velocity-flow integrals and vessel cross-sectional area as previously described.5

### Biochemical Analyses
Serum total cholesterol, triglycerides, HDL cholesterol, and glucose were determined with an automated analyzer (Hitachi Instruments). LDL cholesterol was calculated according to the Friedewald formula.14 Ascorbic acid concentrations were determined in metaphosphoric acid–precipitated plasma that was stored at −70°C as described.11 A blinded post hoc analysis was performed to explore the effect of treatment on markers of lipoprotein oxidation and cellular glutathione with consecutive samples selected at random from each treatment group without regard to clinical characteristics or vascular function. We determined plasma concentrations of 8-epi-prostaglandin F2α, a stable and specific marker of in vivo lipid peroxidation,13 with an ELISA kit (Cayman Corp) and a previously described sample preparation protocol.16 o,o’-Dityrosine and o-tyrosine have been reported to be products of myeloperoxidase- and metal ion–catalyzed LDL oxidation, respectively.17 Dityrosine levels are elevated in LDL isolated from atherosclerotic lesions.1 We measured urine concentrations of these markers with gas chromatography with stable isotope dilution mass spectrometry. Urine samples (1 mL) were supplemented with 10% (vol/vol) trichloroacetic acid and centrifuged at 14,000 rpm for 5 minutes. Then 0.4 mL of urine supplemented with 100 μL of trichloroacetic acid and 18O-labeled internal standards was loaded onto a C18 solid-phase extraction column (3 mL; Supelclean SPE; conditioned as described17). The column was washed with 6 mL of 0.1% trichloroacetic acid. Amino acids were eluted with 3 mL of 10% methanol, concentrated to dryness under vacuum, and then derivatized for analysis.

### Statistical Analyses
Data are presented as mean±SD unless otherwise indicated. Clinical characteristics for the 2 treatment groups were compared by the unpaired t test or the χ² test as appropriate. The effect of treatment on brachial artery diameter, flow-mediated dilation, hyperemic flow, and the biochemical analyses listed above were compared by repeated-measures ANOVA with post hoc Student-Newman-Keuls comparison. Statistical analysis was performed with SigmaStat for Windows 2.03 (SPSS Inc).

### Results
#### Subject Characteristics
Fifty-five subjects were enrolled. Eight were excluded before the data were unblinded because of poor image quality. One subject was excluded because his physician initiated therapy with an ACE inhibitor during the study. The clinical characteristics of the remaining 46 subjects are shown in Tables 1 and 2. There were 4 postmenopausal women in the ascorbic acid group and none in the placebo group (P=0.03). There was a trend toward lower fasting glucose concentration in the placebo group (P=0.10). Otherwise, there were no significant differences in baseline characteristics and no change in these characteristics during treatment.

#### Brachial Artery Responses
As shown in Figure 1, baseline brachial artery flow-mediated dilation was similar in the ascorbic acid (6.6±3.5%) and placebo (8.6±4.7%) groups (P=0.11). Flow-mediated dilation improved 2 hours after a single 2-g dose of ascorbic acid (10.1±5.2%, P<0.05). The improvement was sustained after...
between groups before or after treatment. There were no differences in the extent of improvement in diabetic subjects compared with nondiabetic subjects (data not shown). Ascorbic acid also improved endothelial function in both men and women and in subjects who were and were not receiving lipid-lowering therapy (data not shown).

**Subjects With Abnormal Baseline Flow-Mediated Dilation**

In our previous study, subjects with flow-mediated dilation <5% derived the most benefit from short-term ascorbic acid treatment. In the present study, there were too few subjects in this category for meaningful analysis. Therefore, we examined the subset of 33 subjects (16 placebo, 17 ascorbic acid) with baseline flow-mediated dilation <10%, which represents the mean response for healthy individuals in our laboratory. As shown in Figure 2, flow-mediated dilation improved from 5.2±2.2% to 8.8±5.0% (P<0.05) after single-dose treatment and to 8.5±3.8% (P<0.05) after long-term treatment. In contrast, there was no change in flow-mediated dilation from baseline (6.2±2.3%) after single-dose (5.9±2.9%) or long-term placebo treatment (5.7±2.5%). The response to ascorbic acid was significantly different from the response to placebo (P=0.002). As shown in Figure 2, the response to nitroglycerin was similar in the ascorbic acid and placebo subsets of patients after single-dose treatment (11.5±3.6% and 11.1±6.0%, respectively) and long-term treatment (9.2±4.3% and 12.5±4.7%, respectively). Baseline vessel diameter, blood flow, hyperemic response, and hemodynamic parameters for the 2 groups were similar at baseline and after single-dose and long-term treatment. Subjects with baseline flow-mediated dilation >10% demonstrated no significant improvement after single-dose or long-term treatment (data not shown).

**Ascorbic Acid, Total Glutathione, and Markers of Oxidation**

As shown in Figure 3, plasma ascorbic acid concentrations at baseline were similar in the ascorbic acid and placebo groups (41±13 and 43±19 μmol/L, respectively). Plasma concentration increased to 116±34 μmol/L (P<0.05) 2 hours after administration of 2 g of ascorbic acid and to 95±36 μmol/L (P<0.05) after 30 days of supplementation with 500 mg/d. In the placebo group, the plasma ascorbic acid concentration was unchanged after single-dose (43±20 μmol/L) and long-term (38±20 μmol/L) treatment. By repeated-measures ANOVA, the response to ascorbic acid differed significantly from placebo (P<0.001).

Blood for ascorbic acid concentration was collected 2.6±1.3 hours after the 500-mg dose of ascorbic acid on the day of the follow-up visit. In a subset of 5 subjects assigned to ascorbic acid treatment, blood was collected and the brachial ultrasound was performed at the time of trough rather than peak ascorbic acid level (24 hours after the dose). Plasma ascorbic acid in these subjects was 49±16 μmol/L at baseline and 62±19 μmol/L at 30 days (P=0.08), and flow-mediated dilation was 4.0±3.4% at baseline and 8.4±4.3% after 30 days of treatment (P=0.009). These results suggest that the observed improvement in flow-
mediated dilation in the whole group did not represent a short-term effect of the morning dose of ascorbic acid.

We also examined the effects of long-term ascorbic acid treatment on markers of lipid and protein oxidation and leukocyte glutathione levels in subsets of subjects. As shown in Table 4, there was no evidence that long-term ascorbic acid treatment reduced plasma 8-epi-prostaglandin F$_2$ concentration, urinary o,o'-dityrosine, or o-tyrosine and no evidence that treatment increased leukocyte total glutathione concentration. The study had 80% power ($\alpha=0.05$) to detect a change of 46% for 8-epi-prostaglandin F$_2$, 21% for o,o'-dityrosine, and 50% for leukocyte glutathione.

**Discussion**

This randomized, double-blind, placebo-controlled study demonstrates that a single oral dose of ascorbic acid improves brachial artery flow-mediated dilation in patients with angiographically proven CAD. This beneficial effect is sustained after 1 month of ascorbic acid treatment. The improvement is particularly notable in subjects with less than normal baseline flow-mediated dilation. The effect on vasomotor function could not be attributed to an increase in the stimulus for dilation (hyperemia) or a change in the capacity of the artery to dilate to an exogenous source of NO (nitroglycerin). Thus, the study suggests that single-dose and long-term ascorbic acid treatments improve the production or bioactivity of EDNO in the brachial artery of patients with CAD.

The findings of the present study confirm the findings of our previous study in patients with CAD and extend those findings to long-term treatment. A similar effect of both single-dose and long-term (2 g/d for 1 month) ascorbic acid treatment was recently described in the radial arteries of patients with congestive heart failure. Those improvements were reversed by intra-arterial $N^G$-monomethyl-L-arginine infusion, suggesting a dependence on NO synthesis. Recent studies have also demonstrated improved endothelium-dependent vasodilation after the short-term administration of ascorbic acid.

**Table 3. Brachial Artery and Hemodynamic Parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Placebo</th>
<th>Ascorbic Acid</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>2 h</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>132±18</td>
<td>135±17</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>77±9</td>
<td>78±8</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>61±11</td>
<td>61±13</td>
</tr>
<tr>
<td>Artery diameter, mm</td>
<td>4.5±0.6</td>
<td>4.4±0.6</td>
</tr>
<tr>
<td>Hyperemic flow, % increase</td>
<td>750±340</td>
<td>880±310</td>
</tr>
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</table>

Data are mean±SD. There were no significant differences in these parameters based on treatment, and no significant change was observed after treatment in either group by repeated-measures ANOVA.

**Figure 2.** Effect of short-term and long-term oral ascorbic acid on flow-mediated dilation (top) or nitroglycerin-mediated dilation (bottom) of brachial artery in patients with CAD and baseline flow-mediated dilation <10%. Subjects underwent brachial ultrasound examination at baseline, 2 hours after a single 2-g oral dose of ascorbic acid, and after 1 month of oral 500 mg/d supplementation. Compared with placebo, ascorbic acid improved flow-mediated dilation ($P=0.002$). Ascorbic acid had no effect on nitroglycerin-mediated dilation. Data are presented as mean±SEM and are derived from 17 subjects treated with ascorbic acid and 16 subjects treated with placebo.

**Figure 3.** Effect of ascorbic acid treatment on plasma ascorbic acid concentration in patients with CAD. Plasma ascorbic acid concentration was determined at baseline, 2 hours after a single 2-g oral dose of ascorbic acid, and after 1 month of oral 500 mg/d supplementation. Compared with placebo, ascorbic acid increased plasma ascorbic acid concentration ($P=0.001$). Data are presented as mean±SEM and are derived from 21 subjects treated with ascorbic acid and 25 subjects treated with placebo.
ascorbic acid in patients with hypertension, adult-onset diabetes mellitus, or cigarette smoking or after a high-fat meal. One previous study failed to demonstrate improvement after long-term treatment with a combination of vitamin E, β-carotene, and ascorbic acid (1 g/d for 1 month) in forearm resistance vessels of patients with hypercholesterolemia. This apparently discordant finding may reflect differences in patient population and concurrent therapy and/or differential responses of conduit and resistance vessels.

With regard to mechanism, it has been proposed that ascorbic acid improves EDNO action by scavenging superoxide anion and preventing “inactivation” of NO. This explanation is attractive because atherosclerosis and hypercholesterolemia are linked to excess generation of superoxide, and superoxide reacts with NO and eliminates its biological activity. One caveat lies in the estimation that only supraphysiological concentrations of ascorbic acid could make this mechanism relevant in vivo. Although ascorbic acid scavenges superoxide, the bimolecular rate constant for this reaction (3.3 × 10^9 mol/L^-1 · s^-1) is approximately 10^6 times less than the rate constant for the reaction between superoxide and NO (1.9 × 10^10 mol/L^-1 · s^-1). Assuming an NO concentration of 0.1 to 1 μmol/L adjacent to the endothelial surface, one would predict that an ascorbic acid concentration of 10 to 100 mmol/L would be required to preserve EDNO action. We recently confirmed this prediction using an in vitro model of superoxide-induced endothelial dysfunction. Thus, it is unlikely that extracellular superoxide scavenging is responsible for the observed effects on endothelial function in the present study, in which the peak plasma ascorbic acid concentration was 116 μmol/L. However, it remains possible that this mechanism was operative in previous studies in which intra-arterial infusion produced plasma concentrations of 1 to 10 mmol/L.

Another potential mechanism for the effect of ascorbic acid on endothelial function is inhibition of LDL oxidation. ox-LDL is cytotoxic to endothelial cells and may inactivate NO directly. Moreover, ox-LDL inhibits receptor-dependent NO release from endothelial cells, an effect that is attributable to formation of lysophosphatidylcholine and altered cell membrane signal transduction. In the present study, we found that long-term ascorbic acid treatment did not reduce plasma 8-epi-prostaglandin F_2α or urine α,ω'-dityrosine concentration. As noted above, the study had sufficient power to detect changes of 46% and 21%, respectively, in these markers of lipid and protein oxidation. This power compares favorably with available data. For example, smoking cessation for 2 weeks is associated with a 38% decrease in plasma F_2 isoprostanes. In a preliminary study, we recently demonstrated a 13% reduction in α,ω'-dityrosine after α-tocopherol treatment of healthy adults. Thus, despite the limited sample sizes, we could have detected changes in these markers comparable to those of published data. Clearly, it remains possible that smaller effects were missed or that these systemic markers do not fully reflect events in the vascular wall.

In addition to scavenging superoxide anion or inhibiting LDL oxidation, ascorbic acid could alternatively improve EDNO action by sparing intracellular glutathione, which together with ascorbic acid is the primary regulator of intracellular redox state. A previous study suggested that ascorbic acid 500 mg/d for 2 weeks increased red blood cell glutathione by 47% in healthy subjects. In support of the importance of glutathione for EDNO action, we recently demonstrated that augmenting intracellular glutathione improves brachial artery flow-mediated dilation in patients with CAD. Our present study failed to show an increase in leukocyte total glutathione concentration after ascorbic acid treatment, which argues against this potential mechanism. However, consideration should also be given to the possibility that ascorbic acid increased the ratio of reduced to oxidized glutathione rather than total glutathione, although an investigation of this possibility would pose logistic problems that were beyond the scope of this human study. Another consideration is that leukocyte glutathione levels may not accurately reflect the redox status of the vascular wall.

Our finding that long-term ascorbic acid treatment improves flow-mediated dilation may be clinically relevant, because other interventions that improve endothelial function have been linked to a reduction in cardiovascular events. There is growing evidence that antioxidant status is relevant to the clinical expression of CAD. With regard to ascorbic acid, leukocyte concentrations are significantly reduced in patients with angiographically documented CAD compared with normal subjects. We recently demonstrated that low plasma ascorbic acid levels are associated with the presence of an unstable coronary syndrome. Previous studies have suggested that ascorbic acid may reduce cardiovascular risk by lowering blood pressure or raising HDL cholesterol; however, our randomized, double-blind, placebo-controlled study demonstrated no such effects in patients with CAD. In a population study, low dietary ascorbic acid intake (<50 mg/d) was associated with increased cardiovascular risk. Conversely, other studies have shown no benefit with

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**TABLE 4. Effect of Treatment on Markers of Oxidation and Leukocyte Glutathione**

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<tr>
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<th>Placebo</th>
<th>Ascorbic Acid</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>Baseline</td>
</tr>
<tr>
<td>Plasma 8-epi-prostagland F_2α, pg/mL</td>
<td>85±30 (7)</td>
<td>72±24 (7)</td>
</tr>
<tr>
<td>Urine α,ω'-dityrosine, pmol/nmol creatinine</td>
<td>5.8±1.3 (6)</td>
<td>6.2±1.9 (6)</td>
</tr>
<tr>
<td>Urine α-tirosine, pmol/nmol creatinine</td>
<td>4.8±2.4 (6)</td>
<td>6.1±1.0 (6)</td>
</tr>
<tr>
<td>Leukocyte glutathione, μmol/g protein</td>
<td>6.8±2.9 (9)</td>
<td>6.4±2.4 (9)</td>
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All data are mean±SD (n). There were no significant differences between treatment groups or visits. Numbers in parentheses indicate number of subjects in each group.
increased ascorbic acid intake. Such negative findings have been explained by the failure to consider a sufficiently low intake level, because tissue stores become saturated with an intake of only 100 mg/day. It remains unknown whether ascorbic acid treatment will reduce cardiovascular risk in CAD patients, but the present study suggests that the beneficial effects of such treatment might occur because of improved endothelial function.

The present study has several limitations. First, we examined the brachial artery, and inferences about the coronary circulation must be made with caution. However, a recent study suggested a close relationship between endothelial dysfunction in these 2 vascular beds. Second, on the return visit, we examined brachial artery flow-mediated dilation at the time of peak ascorbic acid concentration. Although we also observed a beneficial effect at the time of trough level in a small subgroup, we do not have such data for the entire group. It is notable that Hornig and colleagues also showed improved endothelial vasomotor function after long-term therapy both before and after a dose of ascorbic acid in patients with congestive heart failure. Third, a number of these subjects with CAD had relatively normal endothelial vasomotor function and would be expected to have little response to an intervention. In addition, there was a nonsignificant trend toward higher flow-mediated dilation in the placebo group. We addressed these issues by examining the subgroup of patients with baseline flow-mediated dilation <10%. In that subgroup, baseline responses were more similar, and as for the group as a whole, ascorbic acid had a highly significant effect. Fourth, although our study was randomized and double-blind, we acknowledge the possibility that some unmeasured confounding variable could have influenced the results. Finally, our exploratory investigation of the effect of ascorbic acid on LDL oxidation was limited by the small sample size and focused on systemic (rather than vascular tissue) markers.

In summary, this randomized, double-blind, placebo-controlled study demonstrates that both single-dose and long-term treatment with ascorbic acid improves endothelial vasomotor function in the brachial artery of patients with CAD. The improvement in function was accompanied by a significant rise in plasma ascorbic acid concentration and a lack of change in blood pressure, lipoprotein profile, or systemic markers of oxidative damage. We speculate that ascorbic acid may improve vasomotor function by enhancing the intracellular redox state or by some other undetermined mechanism. The sustained improvement in endothelial function may, in part, explain the observed epidemiological findings linking ascorbic acid intake with reduction in risk of ischemic heart disease. These results provide further justification for trials investigating ascorbic acid therapy and cardiovascular disease.

Acknowledgments

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