Pharmacological Concentrations of Ascorbic Acid Are Required for the Beneficial Effect on Endothelial Vasomotor Function in Hypertension

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Debra L. Sherman, John F. Keaney, Jr, Elizabeth S. Biegelsen, Stephen J. Duffy, Jay D. Coffman, Joseph A. Vita

Abstract—Increased production of superoxide anion may contribute to impaired bioactivity of endothelium-derived nitric oxide in hypertension. Ascorbic acid is capable of scavenging superoxide anion; however, experimental studies have shown that high physiological concentrations (>1 mmol/L) of ascorbic acid are required to prevent superoxide-mediated vascular dysfunction. To seek kinetic evidence that superoxide anion contributes to endothelial vasomotor dysfunction in human hypertension, we examined the effects of 2.4 or 24 mg/min ascorbic acid intra-arterial infusions on forearm blood flow responses to methacholine or sodium nitroprusside in 30 patients with hypertension and 22 age-matched controls. Endothelium-dependent vasodilation to methacholine was significantly impaired in the hypertensive patients, with a response to the highest dose of methacholine (10 μg/min) of 12.3±6.7 compared with 16.1±5.8 mL·min⁻¹·dL tissue⁻¹ in the controls (P<0.001). The response to sodium nitroprusside was equivalent in the 2 groups. Ascorbic acid at 24 mg/min significantly improved the forearm blood flow response to methacholine in hypertensive patients with a peak response of 16.1±7.1 mL·min⁻¹·dL tissue⁻¹ (P=0.001). This dose produced a cephalic vein ascorbic acid concentration of 3.2±1.4 mmol/L. In contrast, ascorbic acid at 2.4 mg/min had no effect on the methacholine response. Ascorbic acid at both doses had no effect on the vasodilator response to sodium nitroprusside in hypertensive patients or the methacholine response in the controls. These results agree with the predicted kinetics for superoxide anion–mediated impairment of endothelium-derived nitric oxide action. Thus, superoxide anion may contribute to impaired endothelium-dependent vasodilation in patients with hypertension. (Hypertension. 2000;35:936-941.)

Key Words: ascorbic acid ■ endothelium ■ hypertension, essential ■ superoxide

The endothelium controls vascular homeostasis through the release of a number of regulatory substances, including nitric oxide. 1 With one exception, 2 the majority of studies suggests that the bioactivity of endothelium-derived nitric oxide (EDNO) is impaired in essential hypertension. 3–5 Loss of EDNO action may contribute to the pathogenesis of the vascular complications of hypertension, including coronary artery disease and stroke. 6

The bioactivity of nitric oxide is limited by its reaction with superoxide anion to form peroxynitrite. 7 Vascular production of superoxide anion is increased in animal models of hypertension, 8,9 and endothelial vasomotor dysfunction in this setting is reversed with superoxide dismutase. 9 Previous studies have demonstrated that acute intra-arterial administration of ascorbic acid improves EDNO-mediated vasodilation in forearm microvessels of patients with hypertension, 10 diabetes mellitus, 11 or hypercholesterolemia, 12 all conditions associated with increased production of reactive oxygen species. Because ascorbic acid is capable of scavenging superoxide anion, one assumption has been that it improves EDNO action in hypertension and other disease states by this mechanism. Using an in vitro model of superoxide-mediated vascular dysfunction, we recently demonstrated that high physiological concentrations (>1 mmol/L) of ascorbic acid are required to prevent superoxide-mediated impairment of EDNO action. 13 The purpose of the present study was to investigate the dose-dependent effects of ascorbic acid on endothelial vasomotor function in patients with hypertension and to seek kinetic evidence that superoxide anion contributes to endothelial dysfunction in this setting.

Methods

Research Subjects

Patients with a clinical history of essential hypertension (on treatment for hypertension or with diastolic blood pressure >95 mm Hg) and age-matched nonhypertensive volunteers were recruited for study by newspaper advertisement. Patients were excluded if they had a history of cigarette smoking, diabetes mellitus (on hypoglycemic treatment or with fasting glucose >140 mg/dL), hypercholesterolemia (on lipid-lowering treatment or with fasting LDL cholesterol greater than the 75th percentile for age and gender), coronary artery disease, peripheral vascular disease, hormone replacement therapy, or the methacholine response in the controls.

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therapy, or use of antioxidant vitamins (vitamins C or E). All subjects provided informed consent.

Study Protocol
Patients discontinued all medications for at least 48 hours, aspirin for 2 weeks, and alcohol and caffeine for 12 hours before participating in the present study. Patients were studied in the postabsorptive state in a quiet, dimmed, temperature-controlled vascular laboratory (24°C). With the use of sterile conditions and 1% lidocaine local anesthesia, a 20-gauge polyethylene catheter (Arrow International) was inserted into the nondominant brachial artery for measurement of blood pressure and infusion of drugs. After catheter insertion, 5% dextrose in water (Baxter Healthcare Co) was infused at 0.4 mL/min for at least 30 minutes while stable baseline flow and blood pressure conditions were established. Forearm blood flow was measured by venous occlusion plethysmography with calibrated mercury-in-silastic strain gauges and automatic venous-cuff occlusion at 40 mm Hg (Hokanson, Inc). Circulation to the hand was excluded by inflating a wrist cuff to suprasystolic pressure 1 minute before initiation of flow measurements. At least 5 separate measurements were made and averaged for each flow determination. Blood pressure was measured via the arterial catheter by use of a pressure transducer (Maxxim Medical) and physiological recorder (Gould Instrument Systems). Forearm vascular resistance was calculated as the ratio of mean blood pressure to flow.

The following drug infusion protocol was completed: (1) serial 5-minute infusions of methacholine (0.3, 1.0, 3.0, and 10 μg/min; Roche Laboratories) or sodium nitroprusside (0.3, 1.0, 3.0, and 10 μg/min; Elkins-Sinn, Inc), (2) dextrose control for 30 minutes to reestablish control conditions, (3) ascorbic acid (Abbott Laboratories) at 2.4 mg/min or 24 mg/min for 10 minutes, and (4) repeat methacholine or nitroprusside infusions while continuing the ascorbic acid infusion. Forearm blood flow and blood pressure were measured at the end of each infusion. The doses of ascorbic acid were selected to provide a final plasma concentration of ~1 and 10 mmol/L, on the basis of measured baseline blood flow of 2.5 mL·min⁻¹·dL tissue⁻¹, an estimated forearm volume of 1 L, and the assumption that ascorbic acid is excluded from red blood cells during the short-term infusion. The effects of the 2 doses of ascorbic acid on the flow responses to methacholine or nitroprusside were examined on separate days in separate subjects.

Biochemical Analyses
Total cholesterol, HDL cholesterol, triglycerides, glucose, and creatinine were measured with an automated analyzer (Hitachi model 717, Hitachi Instruments). LDL cholesterol was calculated by the Friedewald formula. Ascorbic acid concentration in metaphosphoric acid–precipitated plasma at baseline was measured by high-performance liquid chromatography and electrochemical detection as previously described. Ascorbic acid concentrations were also determined in samples collected from the cephalic vein in the same arm during ascorbic acid infusion in arbitrarily selected patients receiving the 24 mg/min dose of ascorbic acid as an indirect measure of the achieved arterial ascorbic acid concentration.

Statistical Analysis
The effects of ascorbic acid on the forearm blood flow responses to methacholine and nitroprusside were examined by repeated-measures ANOVA with Student-Newman-Keuls post hoc comparison. Clinical characteristics for the hypertensive and normal groups were compared by the 2-tailed unpaired t test or the χ² test as appropriate. We explored the relations between ascorbic acid concentration, conventional risk factors for atherosclerosis, methacholine responses, and improvement after ascorbic acid infusion by use of linear regression analysis. Analyses were performed with the use of SigmaStat for Windows Version 2.03 (SPSS Inc). Data are presented as mean±SD unless otherwise indicated.

Clinical Characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Hypertensive Subjects (N=30)</th>
<th>Normotensive Subjects (N=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>49±12</td>
<td>45±11</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>19 (63%)</td>
<td>12 (55%)</td>
</tr>
<tr>
<td>African American, n (%)</td>
<td>15 (50%)</td>
<td>5 (24%)</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>192±32</td>
<td>184±31</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>44±10</td>
<td>53±19*</td>
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<tr>
<td>LDL cholesterol, mg/dL</td>
<td>118±29</td>
<td>110±31</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>147±103</td>
<td>102±79</td>
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<tr>
<td>Fasting glucose, mg/dL</td>
<td>104±17</td>
<td>93±11*</td>
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<tr>
<td>Serum creatinine, mg/dL</td>
<td>0.9±0.2</td>
<td>0.8±0.2</td>
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<tr>
<td>Plasma renin activity, ng · mL⁻¹ · h⁻¹</td>
<td>1.2±0.9</td>
<td>1.1±1.0</td>
</tr>
<tr>
<td>Plasma aldosterone, ng/dL</td>
<td>9.4±4.5</td>
<td>8.2±4.2</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>148±16</td>
<td>122±13†</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>96±11</td>
<td>78±8†</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>30.7±5.6</td>
<td>26.1±4.7*</td>
</tr>
<tr>
<td>Plasma ascorbic acid, μmol/L</td>
<td>48.3±16.5</td>
<td>60.1±27.8</td>
</tr>
</tbody>
</table>

Values are mean±SD or number (percent), as appropriate. To convert cholesterol concentrations to mmol/L, multiple by 0.02586.

*P<0.05 vs hypertensive group; †P=0.001 vs hypertensive group.

Results

Subjects
A total of 30 hypertensive patients and 22 normotensive subjects were enrolled in the study. Their clinical characteristics are displayed in the Table. The 2 groups were matched for age, gender, and total and LDL cholesterol. As expected, blood pressure was significantly higher in the hypertensive group. In addition, the hypertensive group had significantly higher body mass index and fasting glucose levels and significantly lower HDL cholesterol levels. There also was a trend for higher triglyceride concentrations in the hypertensive group (P=0.07). Baseline ascorbic acid levels were similar in the hypertensive and normotensive patients (P=0.14).

Comparison of Forearm Blood Flow Responses in Normotensive and Hypertensive Subjects
As shown in Figure 1, baseline forearm blood flow was comparable in hypertensive and normotensive groups (2.6±1.1 and 2.5±1.0 mL·min⁻¹·dL tissue⁻¹, respectively). Intra-arterial infusion of methacholine increased forearm blood flow in both groups; however, the vasodilator response was lower in the hypertensive subjects (P<0.001 by repeated-measures ANOVA). The response to the highest dose of methacholine (10 μg/min) was 12.3±6.7 mL·min⁻¹·dL tissue⁻¹ in the hypertensive subjects and 16.1±5.8 mL·min⁻¹·dL tissue⁻¹ in the normotensive subjects. By linear regression analysis, glucose, triglycerides, body mass index, and HDL cholesterol did not correlate with the methacholine response. The vasodilator responses to intra-arterial nitroprusside were equivalent in the 2 groups (Figure 1), with responses to the highest dose (10 μg/min) of 12.4±2.5 and
12.8±4.9 mL·min⁻¹·dL tissue⁻¹, respectively. Blood pressure was not affected by methacholine or nitroprusside at any dose, and forearm vascular resistance paralleled the forearm blood flow responses (data not shown).

### Effect of Ascorbic Acid on Forearm Blood Flow Responses in Hypertensive Subjects

As shown in Figure 2, ascorbic acid infusion in 9 patients at 2.4 mg/min had no effect on the forearm blood flow response to methacholine in hypertensive patients. At peak doses of methacholine, the forearm blood flow responses were 12.7±5.8 and 12.5±5.8 mL·min⁻¹·dL tissue⁻¹ before and during ascorbic acid infusion, respectively. In contrast, ascorbic acid infusion in 12 patients at 24 mg/min improved the forearm blood flow response to methacholine (P<0.001 by repeated-measures ANOVA). At peak methacholine dose, the forearm blood flow response increased from 12.1±7.8 to 16.1±7.1 mL·min⁻¹·dL tissue⁻¹. The response during high-dose ascorbic acid infusion was equivalent to the response of the age-matched normal controls (P=0.93). By linear regression analysis, glucose, triglycerides, and HDL cholesterol did not correlate with the extent of improvement.

As shown in Figure 3, ascorbic acid at 24 mg/min had no effect on the forearm blood flow response to methacholine in the age-matched normal subjects. At peak methacholine doses, the forearm blood flow responses were 15.9±6.8 and 14.4±5.7 mL·min⁻¹·dL tissue⁻¹ before and during ascorbic acid infusion, respectively. Furthermore, this dose of ascorbic acid had no effect on the forearm blood flow response to sodium nitroprusside in hypertensive patients. At peak nitroprusside dose, the forearm blood flow responses were 12.9±5.3 and 12.0±4.2 mL·min⁻¹·dL tissue⁻¹ before and during ascorbic acid infusion, respectively.

During ascorbic acid infusions at 24 mg/min, the ascorbic acid concentration in blood collected from the cephalic vein...
in 9 patients was $3.2 \pm 1.4$ mmol/L. This concentration is compatible with the predicted concentration of 10 mmol/L, because the cephalic vein concentration likely does not fully reflect the local concentration in forearm resistance vessels. There was no correlation between baseline ascorbic acid concentration and the response to methacholine (data not shown). There also was no correlation between baseline ascorbic acid concentration and the extent of improvement in methacholine response (data not shown).

Discussion

The present study demonstrates that the forearm blood flow response to methacholine infusion is impaired in patients with hypertension compared with age-matched controls, whereas the blood flow response to sodium nitroprusside is intact. These findings are consistent with impaired EDNO action in forearm microvessels. Concomitant infusion of ascorbic acid at 24 mg/min produced a high physiological concentration in the forearm circulation (at least 3.2 mmol/L) and restored the methacholine response to normal, whereas infusion of a 10-fold lower concentration had no effect on the methacholine response. Ascorbic acid had no effect on the response to nitroprusside in hypertensive subjects and no effect on the response to methacholine in normal subjects. Thus, the high-dose ascorbic acid infusion reversed endothelial dysfunction in patients with hypertension. Although there was a trend for lower baseline plasma ascorbic acid concentrations in the hypertensive patients, there was no correlation between this parameter and endothelium-dependent dilation at baseline or the extent of improvement during ascorbic acid infusion. These findings suggest that the improvement in endothelial vasomotor function with ascorbic acid cannot be explained by correction of an absolute deficiency of ascorbic acid.

The findings of the present study support the results of previous studies demonstrating impaired forearm blood flow responses to endothelium-dependent vasodilators in patients with hypertension. Although HDL cholesterol concentrations were lower and plasma glucose and triglyceride concentrations were higher in the hypertensive patients compared with the controls, linear regression analysis suggests that these factors did not explain the observed differences in the response to methacholine. The association of these factors with hypertension has been previously recognized.

Previous studies have also demonstrated beneficial effects of high concentrations of ascorbic acid on endothelial vasomotor function in patients with risk factors for coronary artery disease. Ting and colleagues observed improved forearm blood flow responses to methacholine during infusion of ascorbic acid (24 mg/min) in patients with diabetes mellitus and hypercholesterolemia. In a recent study, Taddei et al demonstrated that ascorbic acid at 0.8 to 16 mg·min⁻¹·100 mL forearm tissue (300 mg/min) produced a dose-dependent improvement in forearm blood flow responses to acetylcholine in patients with essential hypertension. In that study, the beneficial effect of ascorbic acid was eliminated by concomitant infusion of N²-monomethyl-L-arginine, confirming its dependence on nitric oxide synthesis. The present study confirms those findings and provides further information about the potential role of superoxide anion as a cause of vascular dysfunction in hypertension.

Previous experimental studies suggest that vascular production of superoxide anion contributes to impaired EDNO action and elevated blood pressure in hypertension. There is evidence that xanthine oxidase and/or angiotensin II–induced NADH/NADPH oxidases may be enzymatic sources of superoxide anion in this disease. Acute elevations in blood pressure and pulsatile stretch of endothelial cells are also associated with increased production of superoxide anion.

Despite this experimental evidence, it has been difficult to confirm a role for superoxide anion in the vascular dysfunction associated with human hypertension. Garcia et al demonstrated no improvement in acetylcholine-mediated dilation of forearm resistance vessels in hypertensive patients during infusion of bovine copper-zinc superoxide dismutase, which reacts with superoxide anion in a highly specific manner. However, failure of this enzymatic preparation to gain access to the intimal or intracellular site of superoxide-nitric oxide interaction might account for these findings. Indeed, in animal studies, beneficial effects of superoxide dismutase are observed only after modification of the enzyme to allow improved access to the endothelial surface. Inhibition of a possible enzymatic source of superoxide anion (xanthine oxidase) also had no effect on endothelial function in hypertensive patients.

Ascorbic acid has the potential to scavenge superoxide anion and prevent formation of peroxynitrite and investi-
superoxide anion contributed to impaired EDNO action in human hypertension, then plasma ascorbic acid concentrations $>1$ mmol/L would be required to improve the forearm blood flow responses to methacholine. The findings of the present study are consistent with this hypothesis: direct measurements in the forearm suggested that the 24-mg/min infusion produces ascorbic acid concentrations in this range. Also consistent are the findings of Taddei et al.,\textsuperscript{10} who observed improved vasodilator responses to acetylcholine during ascorbic acid infusion of $>8$ mg/min (estimated plasma concentration 3.3 mmol/L). Thus, increased production of superoxide anion may account for impaired EDNO action in patients with hypertension, and scavenging of superoxide anion may account for the beneficial effects of ascorbic acid in this setting. However, we acknowledge that ascorbic acid also has activity against a variety of other reactive oxygen species\textsuperscript{22} and that our findings are not specific for superoxide anion.

The observation that the vasodilator response to sodium nitroprusside is preserved in hypertensive patients appears to contradict the conclusion that increased production of superoxide anion accounts for impaired nitric oxide action in hypertension. One would expect that nitric oxide released from sodium nitroprusside should also be susceptible to inactivation by vascular-derived superoxide anion. Because the endothelium has been implicated as a cellular source of superoxide anion,\textsuperscript{23} investigators have argued that nitric oxide produced within endothelial cells might be more susceptible to inactivation than nitric oxide released from nitroprusside.\textsuperscript{10} However, this suggestion would not explain the improved response to EDNO agonists observed after treatment with enzymatic superoxide dismutase in animal models of hypertension.\textsuperscript{20} Ascorbic acid is likely to also be acting extracellularly, in view of the fact that it was ineffective against an endogenous source of superoxide in isolated aorta,\textsuperscript{13} and uptake into endothelial cells is probably minimal during the 20- to 30-minute time course of the study.\textsuperscript{24} As an alternative explanation for preserved nitroprusside responses and the lack of ascorbic acid effect on nitroprusside responses in hypertensive patients, one might consider that nitroprusside and EDNO may produce vasodilation by different mechanisms. In support of this possibility, vasodilator responses to authentic NO are impaired in hypercholesterolemia,\textsuperscript{25} whereas responses to sodium nitroprusside are preserved in this condition, which is known to be associated with increased production of superoxide anion.\textsuperscript{26}

Regarding clinical implications, epidemiological studies suggest links between ascorbic acid status and blood pressure\textsuperscript{27} and cardiovascular disease.\textsuperscript{28} Chronic ascorbic acid treatment has been shown to improve EDNO action in patients with coronary artery disease\textsuperscript{29} and congestive heart failure.\textsuperscript{30} Although it is tempting to conclude that chronic ascorbic acid improves endothelial function by scavenging superoxide anion, it is unlikely that this mechanism is operative with the plasma levels achieved with chronic oral treatment (60 to 100 $\mu$mol/L). Thus, alternative mechanisms for the beneficial effects of chronic oral ascorbic acid should be considered.

In conclusion, the present study demonstrates that high physiological levels of ascorbic acid are required to restore EDNO action in patients with hypertension. These results agree with the predicted kinetics for superoxide anion–mediated impairment of endothelium-derived nitric oxide action. Thus, superoxide anion may contribute to impaired endothelium-dependent vasodilation in patients with hypertension.

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**References**