Oral antioxidant therapy improves endothelial function in Type 1 but not Type 2 diabetes mellitus

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Beckman, Joshua A., Allison B. Goldfine, Mary Beth Gordon, Leslie A. Garrett, John F. Keaney, Jr., and Mark A. Creager. Oral antioxidant therapy improves endothelial function in Type 1 but not Type 2 diabetes mellitus. Am J Physiol Heart Circ Physiol 285: H2392–H2398, 2003.—Oxidative stress decreases the bioavailability of endothelium-derived nitric oxide in diabetic patients. We investigated whether impaired endothelium-dependent vasodilation (EDV) in diabetes can be improved by long-term administration of oral antioxidants. Forty-nine diabetic subjects [26 Type 1 (T1) and 23 Type 2 (T2)] and 45 matched healthy control subjects were randomized to receive oral vitamin C (1,000 mg) and vitamin E (800 IU) daily or matching placebo for 6 mo. Vascular ultrasonography was used to determine brachial artery EDV and endothelium-independent vasodilation (EIV). EDV was increased in both T1 (4.9 ± 0.9%, P = 0.015) and T2 (4.1 ± 1.0%, P < 0.01) subjects compared with control subjects (7.7 ± 0.7%). EIV was decreased in T1 (10.2 ± 0.3%) compared with controls (21.8 ± 1.8%). Administration of antioxidant vitamins increased EDV in T1 (by 3.4 ± 1.4%, P = 0.023) but not T2 subjects (by 0.5 ± 0.4%, P = 0.3). Antioxidant therapy had no significant affect on EIV. Oral antioxidant therapy improves EDV in T1 but not T2 diabetes. These results are consistent with the lack of clinical benefit in studies that have included primarily T2 diabetic patients.

Methods

Subjects. Ninety-five subjects, including 49 with diabetes and 45 matched controls, were recruited through newspaper advertisements and from the Joslin Diabetes Center. Of the diabetic subjects, 26 had Type 1 diabetes and 23 had Type 2 diabetes. All subjects underwent screening medical history, physical examination, and laboratory analysis including complete blood count, serum electrolytes, glucose, glycated hemoglobin, blood urea nitrogen, creatinine, transami-
Vascular reactivity studies. Subjects were studied in a quiet, temperature-controlled, dimly lit room, after resting supine for a minimum of 5 min. High-resolution B-mode ultrasonography of the brachial artery was performed using a Toshiba 270 SSA (Toshiba America Medical Systems; Tustin, CA) ultrasound machine and a 7.5-MHz linear array probe. The brachial artery was imaged longitudinally just proximal to the antecubital fossa. The transducer position was adjusted to obtain optimal images of the near and far wall of the intima. Images were simultaneously recorded on super VHS videotape. The video output and electrocardiographic signal of the ultrasound machine were connected to a computer equipped with a Data Translation frame-grabber videocard (Dataviz; Trumbull, CT). The R-wave on the electrocardiogram was studied in the morning in the postabsorptive state, fasting after the previous midnight. Cyclooxygenase inhibitors, alcohol, and caffeine were prohibited for 24 h before the study. Upon completion of the baseline vascular function studies, subjects were randomized to receive either ascorbate (vitamin C; 1,000 mg, Bronson Laboratories; American Fork, UT) and α-tocopherol (vitamin E; 800 IU, Bronson Laboratories) orally per day or matching placebo. The doses were chosen to maximize antioxidant capacity while limiting adverse events (26, 31). Ninety and 180 days after baseline, subjects underwent repeat vascular function testing.

Vascular reactivity studies. Subjects were studied in a quiet, temperature-controlled, dimly lit room, after resting supine for a minimum of 5 min. High-resolution B-mode ultrasonography of the brachial artery was performed using a Toshiba 270 SSA (Toshiba America Medical Systems; Tustin, CA) ultrasound machine and a 7.5-MHz linear array probe. The brachial artery was imaged longitudinally just proximal to the antecubital fossa. The transducer position was adjusted to obtain optimal images of the near and far wall of the intima. Images were simultaneously recorded on super VHS videotape. The video output and electrocardiographic signal of the ultrasound machine were connected to a computer equipped with a Data Translation frame-grabber videocard (Dataviz; Trumbull, CT). The R-wave on the electrocardiogram served as a trigger to acquire frames at end diastole. After baseline image acquisition, a forearm sphygmomanometric cuff was inflated to suprasystolic pressure (200 mmHg) for 5 min. Upon cuff release, reactive hyperemia causes flow to increase through the brachial artery subserous to the forearm. Flow-induced, endothelium-dependent vasodilation of the brachial artery was determined by acquiring images at 1 min after cuff deflation. Flow-mediated vasodilation at this time point is largely endothelium dependent and NO mediated and can be inhibited by the administration of the NOS (NO synthase) antagonist Nω-nitro-L-arginine (32). Ten minutes after cuff release, the brachial artery was imaged again to reestablish basal conditions. To determine endothelium-independent vasodilation, subjects then received 0.4 mg of nitroglycerin sublingually. The brachial artery was imaged 3 min later. Brachial artery blood flow velocity was determined via time-velocity integral measurement. Nitroglycerin was not administered if the systolic blood pressure was below 110 mmHg or if the subject refused nitroglycerin, usually to avoid a severe headache during the second and third visits. Fifty-three of the 94 subjects received nitroglycerin.

Laboratory analyses. Vitamin C and E levels were measured at baseline and at 3 mo in a subset of 18 diabetic and 25 healthy control subjects. Also, the lag time of copper-induced oxidation of LDL was measured at baseline and at 3 mo. Vitamin E reaches its maximal antioxidant effect at 3 mo. The effect is maintained to 1 yr with continued therapy (38).

Blood was collected into Vacutainer tubes (Becton Dickinson) containing 286 USP units sodium heparin/15 ml whole blood and maintained at 4°C protected from light until the plasma was prepared by centrifugation (1,200 g) at 4°C for 15 min. For vitamin E analysis, plasma was stored at −70°C. Plasma vitamin E levels were analyzed using reverse-phase HPLC with electrochemical detection as described previously (37). For vitamin C determination, plasma was precipitated with an equal volume of 5% (wt/vol) metaphosphoric acid, and the supernatant was stored at −70°C. Plasma vitamin C levels were determined using paired-ion reverse-phase HPLC (14). For isolation of LDL, plasma was immediately subjected to single vertical spin discontinuous density gradient ultracentrifugation (46). For measurement of LDL susceptibility to oxidation, incubations contained 100 μg LDL protein in 1 ml PBS and a final concentration of 3.3 μmol/l CuCl2 or 4.0 mmol/l 2,2'-azo-bis(2-aminopropane) dihydrochloride (Eastman Kodak). LDL oxidation was monitored by conjugated diene formation at 57°C in a Varian Cary 3 spectrophotometer, and the duration of the lag phase was calculated as previously described (46).

Statistical methods. Descriptive measures are reported as means ± SD. Experimental measures are reported as means ± SE. Baseline vitamin levels and lag-phase oxidation were compared using nonpaired two-tailed t-tests. Demographic data, arterial diameter, reactive hyperemia, and flow-mediated and nitroglycerin-mediated vasodilation were compared using ANOVA and a post hoc Tukey’s test. Two-way repeated-measures ANOVA was performed to compare the effect of vitamins and placebo on flow-mediated and nitroglycerin-mediated vasodilation. The effect of vitamin supplementation on vitamin levels and lag-phase oxidation were compared using ANOVA. Multiple linear regression was performed to assess the effects of baseline characteristics on the primary end point. Statistical significance was accepted at the 95% confidence level (P < 0.05). All statistics were run on SPSS Base 10.0 (SPSS; Chicago, IL).

RESULTS

Baseline characteristics are presented in Table 1. Subjects with diabetes and healthy controls were well matched by age and sex. When separated into groups by diabetes type, predictable differences among the three groups were noted in age, body mass index, and total cholesterol. By post hoc Bonferroni analysis, sub-

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Values are means ± SE, n, no. of subjects. BMI, body mass index; LDL, low-density lipoprotein; MAP, mean arterial pressure; hemoglobin A₁C, glycosylated hemoglobin. *P < 0.05 and †P < 0.01 vs. control and Type 1 diabetes values.
subjects with Type 2 diabetes were older and had a greater body mass index, higher mean arterial pressure, and modestly increased total cholesterol compared with subjects with Type 1 diabetes and healthy controls (all \( P < 0.05 \)). In contrast, subjects with Type 1 diabetes had a longer duration of disease, 11.8 \( \pm \) 2.8 yr, compared with Type 2 subjects, 7.5 \( \pm \) 2.1 yr (\( P = 0.005 \)).

Vitamin levels and oxidative stress. At baseline, plasma vitamin C levels were significantly lower in diabetic subjects compared with healthy control subjects (30.8 \( \pm \) 1.1 vs. 45.2 \( \pm \) 1.3 \( \mu \)mol/l, \( P = 0.02 \)). Administration of vitamin C significantly increased plasma vitamin concentrations in both diabetic subjects and healthy control subjects. In diabetic subjects that received vitamin C, plasma levels increased from 30.6 \( \pm \) 2.2 to 71.0 \( \pm \) 2.2 \( \mu \)mol/l (\( P < 0.01 \)), whereas the vitamin C concentration did not change significantly in those subjects that took the placebo (from 31.1 \( \pm \) 1.6 to 33.8 \( \pm \) 1.5 \( \mu \)mol/l, \( P = 0.09 \)). In healthy subjects who received vitamin C, plasma levels increased from 47.0 \( \pm \) 1.1 to 72.8 \( \pm \) 4.0 \( \mu \)mol/l (\( P < 0.01 \)), whereas in those who received the placebo, the vitamin C concentration did not change (from 43.5 \( \pm \) 3.1 to 43.0 \( \pm \) 4.9 \( \mu \)mol/l, \( P = 0.3 \)).

At baseline, plasma vitamin E levels were similar between diabetic and healthy control subjects (2.3 \( \pm \) 2.4 mg/dl, \( P = 0.4 \)). Administration of vitamin E significantly increased plasma vitamin concentrations in diabetic subjects and healthy control subjects. In diabetic subjects who received vitamin E, plasma levels increased from 2.3 \( \pm \) 0.4 to 4.6 \( \pm \) 0.7 mg/dl (\( P = 0.04 \)), whereas in those who received the placebo, the vitamin E concentration did not change (from 2.4 \( \pm \) 0.3 to 2.5 \( \pm \) 0.4 mg/dl, \( P = 0.7 \)). In control subjects who received vitamin E, plasma levels increased from 2.2 \( \pm \) 0.2 to 3.9 \( \pm \) 0.3 mg/dl (\( P < 0.01 \)), whereas in those who received the placebo, it did not change (2.2 \( \pm \) 0.3 to 2.0 \( \pm \) 0.2 mg/dl, \( P = 0.3 \)).

The lag-phase copper oxidation of LDL was measured to determine the effect of vitamin administration on oxidative stress. Lag time was similar between diabetic and healthy control subjects at baseline (71.8 \( \pm \) 3.0 vs. 68.5 \( \pm \) 2.8 min, \( P = 0.24 \)). Antioxidant vitamin administration increased lag time in both groups, from 74 \( \pm \) 5.4 to 89 \( \pm \) 6.9 min (\( P = 0.19 \)) in diabetic subjects and from 67.2 \( \pm \) 3.9 to 84.4 \( \pm \) 4.0 min (\( P < 0.01 \)) in control subjects. There was no significant difference in lag time between Type 1 and Type 2 subjects who received vitamins (86.8 \( \pm \) 10 vs. 91.5 \( \pm \) 27 min, \( P = 0.8 \)). Lag time did not change significantly in healthy control or diabetic subjects who received the placebo (from 69 \( \pm \) 9 to 71 \( \pm \) 17 min, \( P = 0.56 \), and from 69 \( \pm \) 15 to 76 \( \pm \) 14 min, \( P = 0.17 \), respectively).

Baseline vascular function. Baseline brachial artery diameter was 3.13 \( \pm \) 0.08 mm in Type 1 subjects, 3.69 \( \pm \) 0.12 mm in Type 2 subjects, and 3.56 \( \pm \) 0.09 mm in control subjects (\( P = 0.002 \) by ANOVA). Baseline diameter was significantly less in Type 1 subjects than control subjects by post hoc testing (\( P = 0.007 \)). Baseline time-averaged blood flow velocity was 7.6 \( \pm \) 0.8, 9.1 \( \pm \) 1, and 10.0 \( \pm \) 1 cm/s in Type 1, Type 2, and control subjects, respectively (\( P = 0.24 \)). The blood flow velocity response to the ischemia stimulus differed across the three groups: 567 \( \pm \) 62% for Type 1 subjects, 319 \( \pm \) 43% for Type 2 subjects, and 463 \( \pm \) 46% for control subjects (\( P = 0.014 \)); post hoc testing revealed no significant differences between either diabetic group and control subjects but greater responses in Type 1 than Type 2 subjects. Flow-mediated vasodilation was significantly decreased in both Type 1 (4.9 \( \pm \) 0.9%, \( P = 0.015 \)) and Type 2 (4.1 \( \pm \) 1%, \( P < 0.01 \)) subjects compared with control subjects (7.7 \( \pm \) 0.7%) (Fig. 1). Nitroglycerin-mediated vasodilation was significantly decreased in Type 2 (15.0 \( \pm \) 1.2%, \( P < 0.01 \)) but not Type 1 subjects (18.5 \( \pm \) 2.3%, \( P = 0.3 \)) compared with control subjects (21.8 \( \pm \) 1.8%) (Fig. 2).

Antioxidant vitamin therapy and endothelium-dependent vasodilation. Basal brachial artery diameter did not vary significantly through the course of the study in any subgroup except Type 1 diabetic subjects randomized to vitamin therapy, in whom it increased...
from 3.06 ± 0.3 to 3.22 ± 0.3 mm (*P = 0.04). The increase in the blood flow velocity response to reactive hyperemia did not change significantly within any group during the study. There was no change in endothelium-dependent vasodilation in healthy, control subjects with or without antioxidant vitamin therapy (Fig. 3). In contrast, antioxidant vitamin therapy increased endothelium-dependent vasodilation in all diabetic subjects by 2.1 ± 0.8%, from 3.4 ± 0.8% to 5.4 ± 0.8% over the 180 days (P = 0.025), whereas placebo had no effect (−0.9% ± 1.2%, from 5.8 ± 1.1% to 4.9 ± 0.9%, P = 0.59) (Fig. 3).

Although antioxidant vitamin treatment increased endothelium-dependent vasodilation in the diabetic group, when considered in total, different responses were found when the Type 1 and Type 2 subjects were evaluated independently. In subjects with Type 1 diabetes, antioxidant administration increased endothelium-dependent vasodilation by 3.4 ± 1.4%, from 4.2 ± 1.3 to 7.6 ± 1.2% (P = 0.023) (Fig. 4), whereas placebo had no effect (0%, from 5.7 ± 1.2 to 5.7 ± 1.5%, P = 0.98). In Type 2 diabetic subjects, treatment with antioxidant vitamins did not improve endothelium-dependent vasodilation (0.5 ± 0.4%, from 2.3 ± 0.9% to 2.8 ± 1.4% P = 0.3) (Fig. 3). Placebo therapy did not affect endothelium-dependent vasodilation in Type 2 subjects (−1.4 ± 2.1%, from 6.3 ± 1.9 to 4.9 ± 1.0%, P = 0.51).

Antioxidant vitamin therapy and endothelium-independent vasodilation. There was no improvement in endothelium-independent vasodilation in Type 1 and Type 2 subjects compared with healthy controls subjects during treatment with either placebo or antioxidant vitamins. Multivariate testing excluded any significant impact of age, sex, body mass index, total cholesterol, LDL-cholesterol, high-density lipoprotein-cholesterol, mean arterial pressure, glucose, and hemoglobin A1C on baseline endothelium-dependent or -independent vasodilation or on the response to oral antioxidants.

**DISCUSSION**

Flow-mediated, endothelium-dependent vasodilation of a peripheral conduit artery is impaired in patients with both Type 1 and Type 2 diabetes mellitus, as reported previously (6, 44). We report two important new findings in this investigation. Nitroglycerin-mediated, endothelium-independent vasodilation is abnormal in conduit arteries of patients with Type 2 diabetes mellitus. Second, oral antioxidant therapy with vitamin C and vitamin E improves endothelium-dependent vasodilation in subjects with Type 1 but not Type 2 diabetes mellitus.

**Endothelial dysfunction and oxidative stress in diabetes.** Decreased endothelium-dependent vasodilation, and, by extension, endothelium-derived NO, has been demonstrated in vitro, in experimental animal models, and in human resistance arterioles with Type 1 and Type 2 diabetes (18, 27, 40, 53). An important contributor to the decrease in bioavailable NO is the heightened production of oxygen-derived free radicals, primarily superoxide anion (10). Increased markers of oxidative stress, including urinary F2-isoprostanes and thiobarbituric acid-reacting species, have been demonstrated in subjects with Type 1 and Type 2 diabetes mellitus (1, 9). Recently, the endothelium has been identified as an important vascular source of superoxide anion in human diabetic vessels (8, 10, 19, 22, 23, 43). In previous proof of principle investigations, supraphysiological intra-arterial infusions of vitamin C, capable of scavenging extracellular superoxide anion (25), restored endothelium-dependent vasodilation in resistance arterioles of subjects with both Type 1 and Type 2 diabetes (50, 53), linking increased oxidative stress to endothelial dysfunction. Indeed, increases in the production of superoxide anion decrease bioavailable NO through a diffusion-limited reaction with NO to form peroxynitrite (3), a weak vasodilator (34). Peroxynitrite enhances the production of superoxide anion by oxidizing the endothelial NOS (eNOS) cofactor tetrahydrobiopterin (41). Peroxynitrite and a relative NO deficiency both antagonize prostacyclin synthase (52, 56). Furthermore, peroxynitrite antagonizes endothelium-derived hyperpolarizing factor-mediated vasodi-
lation (35). Thus the observed impairment in endothelium-dependent vasodilation may also result as a consequence of the downstream effects of increased oxidative stress and decreased NO bioavailability on the production of multiple endothelium-derived mediators.

**Measures of oxidative stress.** Baseline vitamin C levels were lower in our subjects with diabetes than healthy control subjects, indicative of increased oxidative stress, confirming previous reports (47). There were no differences in vitamin E levels between our diabetic and healthy control groups. In subjects with diabetes, vitamin E levels have been reported as both increased (28) and not different from controls (5). Additionally, there was no significant difference in time to copper-induced LDL oxidation between diabetic and healthy control subjects in our study. In a recent study (11) examining F$_2$-isoprostanes and LDL oxidation time as measures of oxidative stress in diabetes, only F$_2$-isoprostanes differed significantly from healthy control subjects. LDL oxidation time is performed on LDL isolated from plasma and may not fully reflect oxidative stress within the vascular wall. Thus, had we measured F$_2$-isoprostanes, we may have found differences in oxidative stress. Also, our findings may have been affected by the requirement of an overnight fast. In one report (12), there was no difference in LDL oxidation times in the fasting state between diabetic and healthy control groups. In subjects with Type 2 diabetes, endothelium-dependent vasodilation did not change significantly with oral antioxidant vitamins. Several important distinctions exist between the two types of diabetes and may explain these findings. Type 2 subjects manifest metabolic abnormalities, including insulin resistance and excess free fatty acid liberation, that may impair endothelium-dependent vasodilation by mechanisms other than increased oxidative stress.

Each of these metabolic disturbances may independently downregulate eNOS production of NO. Insulin resistance increases asymmetric dimethylarginine concentrations, an endogenous inhibitor of eNOS, and augments Ras and Rho kinase activation, enzymes associated with decreased eNOS content and activity (42, 49). Excess free fatty acid concentrations inhibit phosphatidylinositol 3-kinase and reduce Akt-related serine phosphorylation of eNOS, thereby decreasing NO production and endothelium-dependent vasodilation (13, 36). Additionally, in the present study, endothelium-independent vasodilation was impaired only in Type 2 patients. This finding is consistent with that of our previous investigations (27, 53) in forearm resistance vessels of patients with Type 1 and Type 2 diabetes. A modest defect in endothelium-independent vasodilation has been described in Type 1 subjects in a study (6) that included 80 Type 1 subjects; our study size does not have the statistical power to exclude this possibility, but the effect was less than that seen in Type 2 subjects. Thus, in Type 2 subjects, the severe dysmetabolism prevents an increase in NO bioavailability or diminished vascular smooth muscle function makes the vessel incapable of responding to an increase in endothelium-derived NO.

Our data conflict with the observations by Paolisso and colleagues (44), who randomly assigned Type 2 diabetic subjects to receive either vitamin E (600 mg/day) or placebo for 8 wk. Similar to the present study, markers of oxidative stress decreased over the duration of therapy. In contrast, conduit-vessel endothelium-dependent vasodilation increased significantly at the 8-wk time point. This group did not evaluate endothelium-independent vasodilation or include healthy control subjects, limiting interpretation of the results. Consistent with our findings in conduit vessels, Gazis and colleagues (15) randomly assigned Type 2 diabetic and healthy control subjects to receive vitamin E (1,600 IU/day) for 8 wk and found that antioxidant therapy did not affect endothelium-dependent or endothelium-independent vasodilation in resistance vessels in diabetic or healthy subjects.
Endothelium-independent vasodilation. There was no difference in endothelium-independent vasodilation in response to antioxidant therapy and placebo between healthy control and diabetic subjects. These results are similar to our investigations (50, 51) using parenteral vitamin C administration in resistance arteries of patients with diabetes.

Clinical implications. A motivation for conducting this investigation was the striking discordance between the positive results of acute and short-duration studies of antioxidants on vascular function and the negative studies in thousands of patients demonstrating no clinical benefit when treated chronically with oral antioxidant vitamins (7, 16, 50, 51, 55). We found that oral antioxidant therapy with vitamins C and E improves vascular function in Type 1 but not Type 2 diabetes. The results in Type 2 patients likely hold the key to understanding the results in large clinical trials of atherosclerosis and diabetes. The more extensive vascular dysfunction and confounding metabolic abnormalities that impair endothelial function in Type 2 diabetes may preclude a chronic benefit from oral antioxidant vitamins. Thus our data provide insight into why vitamins are unable to provide clinical benefit in large clinical trials: the vast majority of diabetic subjects in these trials had Type 2 diabetes.

DISCLOSURES

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