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High-dose oral vitamin C partially replenishes vitamin C levels in patients with Type 2 diabetes and low vitamin C levels but does not improve endothelial dysfunction or insulin resistance

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[1]*Diabetes Unit, National Center for Complementary and Alternative Medicine;* [2]*Cardiology Branch, National Heart, Lung, and Blood Institute;* [3]*Molecular and Clinical Nutrition Section, Digestive Diseases Branch, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland*

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Chen, Hui, Rajaram J. Karne, Gail Hall, Umberto Campia, Julio A. Panza, Richard O. Cannon III, Yaohui Wang, Arie Katz, Mark Levine, and Michael J. Quon. High-dose oral vitamin C partially replenishes vitamin C levels in patients with Type 2 diabetes and low vitamin C levels but does not improve endothelial dysfunction or insulin resistance. *Am J Physiol Heart Circ Physiol* 290: H137–H145, 2006. First published August 26, 2005; doi:10.1152/ajpheart.00768.2005.—Endothelial dysfunction is a hallmark of Type 2 diabetes related to hyperglycemia and oxidative stress. Nitric oxide-dependent vasodilator actions of insulin may augment glucose disposal. Thus endothelial dysfunction may worsen insulin resistance. Intra-arterial administration of vitamin C improves endothelial dysfunction in diabetes. In the present study, we investigated effects of high-dose oral vitamin C to alter endothelial dysfunction and insulin resistance in Type 2 diabetes. Plasma vitamin C levels in 109 diabetic subjects were lower than healthy (36 ± 2 μM) levels. Thirty-two diabetic subjects with low plasma vitamin C (<40 μM) were subsequently enrolled in a randomized, double-blind, placebo-controlled study of vitamin C (800 mg/day for 4 wk). Insulin sensitivity (determined by glucose clamp) and forearm blood flow in response to ACh, sodium nitroprusside (SNP), or insulin (determined by plethysmography) were assessed before and after 4 wk of treatment. In the placebo group ($n = 17$ subjects), plasma vitamin C (22 ± 3 μM), fasting glucose (159 ± 12 mg/dl), insulin (19 ± 7 μU/ml), and SI_{Clamp} [2.06 ± 0.29 × 10^{-4} dl·kg^{-1}·min^{-1}/(μU/ml)] did not change significantly after placebo treatment. In the vitamin C group ($n = 15$ subjects), basal plasma vitamin C (23 ± 2 μM) increased to 48 ± 6 μM ($P < 0.01$) after treatment, but this was significantly less than that expected for healthy subjects (>80 μM). No significant changes in fasting glucose (156 ± 11 mg/dl), insulin (14 ± 2 μU/ml), SI_{Clamp} [2.71 ± 0.46 × 10^{-4} dl·kg^{-1}·min^{-1}/(μU/ml)], or forearm blood flow in response to ACh, SNP, or insulin were observed after vitamin C treatment. We conclude that high-dose oral vitamin C therapy, resulting in incomplete replenishment of vitamin C levels, is ineffective at improving endothelial dysfunction and insulin resistance in Type 2 diabetes.

hypertension; hyperglycemia; hypercholesterolemia; insulin sensitivity; sodium nitroprusside

TYPE 2 DIABETES is characterized by both endothelial dysfunction and insulin resistance (14, 20). Vascular actions of insulin stimulating production of nitric oxide (NO) from endothelium (81) lead to vasodilation and increased blood flow to skeletal muscle that significantly augment insulin-mediated glucose disposal (3–5, 7, 64). Thus endothelial dysfunction may directly contribute to insulin resistance. Conversely, insulin resistance may contribute to endothelial dysfunction because signaling pathways mediating metabolic actions of insulin in skeletal muscle and adipose tissue are strikingly similar to those regulating insulin-stimulated production of NO in vascular endothelium (34, 49–51, 78, 81, 82). Indeed, impaired vasodilator actions of insulin in obese and diabetic subjects are positively correlated with metabolic insulin resistance (40, 72).

The hyperglycemia of diabetes worsens both endothelial dysfunction and insulin resistance by multiple independent mechanisms including increased oxidative stress that accelerates the inactivation of endothelium-derived NO (8, 13, 29, 55, 80). Patients with diabetes or the metabolic syndrome have low levels of the antioxidant vitamin C (24, 67, 79). Acute intra-arterial administration of pharmacological doses of vitamin C improves endothelial dysfunction in subjects with diabetes, obesity, hypertension, hypercholesterolemia, or acute hyperglycemia (9, 54, 61, 65, 73–76). Intravenous infusion of pharmacological doses of vitamin C in diabetic subjects increases renal perfusion (21). However, in hypertensive subjects, improvement of brachial artery endothelial function with intra-arterial vitamin C is not associated with improvement in insulin-mediated glucose uptake in the forearm (54). In addition to direct antioxidant effects, vitamin C may ameliorate hyperglycemia-induced oxidative stress in the endothelium by regulating prooxidant enzyme activity (77). However, neither intra-arterial nor intravenous administration of vitamin C is a practical clinical therapy.

It is controversial whether oral vitamin C supplementation in humans is beneficial. In several epidemiological studies, an inverse correlation between vitamin C intake and mortality is observed (33, 34). Although acute oral vitamin C therapy improves vascular function in patients with coronary artery disease (42) and chronic oral vitamin C therapy improves endothelial function in children with hyperlipidemia (23), chronic supplementation with oral vitamin C is not associated with decreases in 5-yr mortality, coronary artery disease, carotid intima-media thickness, blood pressure, or diabetic hyperlipidemia (2, 12, 29a, 31, 35). In some studies, chronic oral administration of vitamin C to patients with Type 2 diabetes causes a decline in plasma free radicals that is associated with

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improved whole body glucose disposal (60), decreased blood pressure (53), and improved endothelial function (62). However, other studies (10, 19) are unable to document the beneficial effects of oral vitamin C supplementation on blood pressure, oxidative stress, and endothelial function in Type 2 diabetes. One recent report (41) suggests that supplemental vitamin C may actually increase the risk of cardiovascular disease in women with diabetes. Because there is no consensus on the beneficial effects of vitamin C supplementation in Type 2 diabetes and because endothelial dysfunction is coupled with insulin resistance, we conducted a randomized, double-blind, placebo-controlled trial of high-dose oral vitamin C (800 mg/day for 4 wk) to evaluate whether this simple therapy may be beneficial in subjects with Type 2 diabetes with respect to vascular and metabolic functions.

METHODS

Study subjects. One hundred nine subjects with Type 2 diabetes between the ages of 23 and 65 yr underwent screening for inclusion in our clinical study (*study 1*). All subjects met the American Diabetes Association criteria for Type 2 diabetes (1) and had their blood drawn for measurement of plasma vitamin C levels. Subjects were excluded from further study if they were on insulin therapy or if they had severe systemic diseases, known atherosclereosis, disease predisposing to vasculitis or Raynaud's phenomenon, bleeding disorders, history of kidney stones, glucose-6-phosphate dehydrogenase deficiency, infection with HIV or hepatitis B, or were pregnant. Diabetic subjects with hypertension whose blood pressure exceeded 170/109 mmHg off of their antihypertensive medication were also excluded from further study. This cutoff of 170/109 mmHg was chosen for safety reasons. Screened subjects from *study 1* whose plasma vitamin C levels were lower than 40 μM and whose fasting blood glucose did not exceed 300 mg/dl off of their antidiabetic medication were invited to enroll in the complete study (*study 2*). Out of 109 screened subjects, 37 subjects enrolled and 32 subjects completed all phases of *study 2*. In addition to having diabetes, 9 out of the 32 subjects who completed *study 2* had essential hypertension. The enrolled subjects were then randomized to receive daily oral doses of either vitamin C (800 mg/25 ml of an aqueous solution) or placebo (500 mg citric acid/25 ml, pH 7.0) for 4 wk in a double-blind fashion. Citric acid was used in the placebo because its taste is indistinguishable from that of ascorbic acid. Subjects were advised to avoid dietary or medicinal intake of vitamin C during the study. All antidiabetic and antihypertensive medications were stopped for 1 wk before subjects were assessed at the beginning and the end of treatment with either vitamin C or placebo. Thus subjects had their medication withdrawn for 1 wk before each glucose clamp and forearm blood flow (FBF) study. Each subject underwent FBF studies and hyperinsulinemic isoglycemic glucose clamp on the same day before and after 4 wk of treatment with either placebo or vitamin C. Informed consent was obtained from each subject. The study protocol was approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute, and all procedures followed were in accordance with institutional guidelines. All studies were conducted in the Clinical Center at the National Institutes of Health.

Plasma vitamin C measurement. Plasma vitamin C levels were measured at the initial screening. Fasting plasma vitamin C levels were measured again immediately before and after a 4-wk administration of vitamin C or placebo. Blood was collected in plasma separator tubes with lithium heparin. Plasma was mixed with 90% methanol and 1 mM EDTA in HPLC-grade water (1:4, vol/vol) and centrifuged at 1,000 *g* at 4°C for 10 min. The supernatant was stored at −70°C until the time of assay. For each sample, three aliquots were analyzed separately by HPLC with coulometric electrochemical detection as described previously (22, 43).

FBF measurement. On study days, brachial artery and antecubital vein catheters were placed in the forearm of each subject at ~7:30 AM after an overnight fast of at least 10 h. Twenty minutes after the placement of the catheters, baseline arterial and venous blood samples were obtained and FBF was measured by silastic strain-gauge venous-occlusion plethysmography, as described previously (15, 59). Endothelium-dependent and -independent vascular function were assessed by measuring FBF in response to graded intra-arterial infusions of ACh and sodium nitroprusside (SNP), respectively. Graded infusion rates were 7.5, 15, and 30 μg/min for ACh, and 0.8, 1.6, and 3.2 μg/min for SNP with a washout period of 20 min between the two drugs. Seven blood flow measurements conducted at 15-s intervals for each drug dose were determined, and the mean blood flow determination for each drug dose was used for further data analysis. The effect of insulin on blood flow was assessed by measuring FBF at baseline, 30, 60, 120, and 180 min after initiation of the insulin infusion during the glucose clamp procedure. Blood pressure was simultaneously determined by using a pressure transducer connected to the intra-arterial cannula. Forearm vascular resistance (FVR) was calculated by dividing mean arterial blood pressure by FBF values. Heart rate was recorded from an electrocardiographic lead. Blood flow data were measured and analyzed by using an EC6 Strain Gauge Plethysmograph with NIVP3 Arterial Inflow Software (Hokanson, Bellevue, WA).

Hyperinsulinemic isoglycemic glucose clamp. The glucose clamp study, conducted immediately after forearm plethysmography with ACh and SNP infusions, was completed according to methods previously described (33). An insulin solution (Humulin, Eli Lilly) was infused into a peripheral arm vein at a rate of 120 $mU \cdot m^{-2} \cdot min^{-1}$ for 3 h using a calibrated syringe pump (model A-99, Razel Industries, Stamford, CT). Potassium phosphate was infused at the same time (0.23 $meq \cdot kg^{-1} \cdot h^{-1}$) to prevent hypokalemia. Whole blood glucose concentrations were measured at the bedside every 5 min using a glucose analyzer (YSI 2700 Select, Yellow Springs Institute, Yellow Springs, OH), and an infusion of 20% dextrose was adjusted to maintain the blood glucose concentration at the fasting level. In addition, blood samples were collected at −10, −5, and 0 min (before starting insulin infusion) to obtain average baseline insulin levels. After the insulin infusion was started, blood samples were collected at 30, 60, 90, 110, 130, 150, and 180 min to measure plasma insulin concentrations (DPC Immulite 2000, Diagnostic Products, Los Angeles, CA). The steady-state period of the clamp was defined as a ≥60-min period, where the coefficient of variation for blood glucose, plasma insulin, and glucose infusion rate was <5%. Mean values during the steady-state period were used to calculate SI_{Clamp}, a glucose clamp derived index of insulin sensitivity. SI_{Clamp} was defined as $M/(G \times \Delta I)$ corrected for body weight, where M is steady-state glucose infusion rate (mg/min), G is steady-state blood glucose concentrations (mg/dl), and ΔI is difference between basal and steady-state plasma insulin concentration (μU/ml).

Alternative indexes of insulin sensitivity. In addition to SI_{Clamp}, we also calculated surrogate indexes, the quantitative insulin-sensitivity check index (QUICKI) (33), and the homeostasis model assessment (HOMA) (46). QUICKI was calculated as $QUICKI = 1/[\log(I_0) + \log(G_0)]$, where I_0 is fasting insulin (μU/ml) and G_0 is fasting glucose (mg/dl). HOMA was calculated as G_0 (mmol/l) × I_0 (μU/ml)/22.5.

Statistical analyses. Changes in various parameters after treatment within a treatment group (placebo or vitamin C) were evaluated by using paired Student's *t*-tests where appropriate. Comparisons between parameters in the placebo and vitamin C group at baseline and after treatment were evaluated by using unpaired Student's *t*-tests. Changes in dose-response curves for FBF or FVR due to treatment with vitamin C or placebo and between placebo and vitamin C groups were analyzed by repeated-measures ANOVA. All data are means ± SE. All *P* values reported represent two-tail analysis. *P* values of <0.05 were considered statistically significant. Study sample size was based on power calculations predicting that our sample size is ade-

Table 1. *Study 1: clinical characteristics of subjects with Type 2 diabetes who were screened for inclusion in the protocol*

Sex, M/W	Age, yr	Vitamin C, μM	BMI, kg/m^2	Random Glucose, mg/dl	HbAlc, %	Duration of Diabetes, yr	BP, mmHg	HR, beats/min	Cholesterol, mg/dl	LDL, mg/dl	HDL, mg/dl	TG, mg/dl
45/64	50±1	36±2	35±1	165±7	7.6±0.2	5±0.5	140±2/77±1	80±1	196±4	123±4	49±1	166±12

Values are means ± SE; n = 109 subjects. M and W, men and women, respectively; BMI, body mass index; HbAlc, hemoglobin-Alc; BP, blood pressure (values given are systolic and diastolic BP, respectively); HR, heart rate; TG, triglycerides.

quate to detect a 10% change in endothelial function and insulin sensitivity with more than 90% power.

RESULTS

Study subjects. Clinical characteristics of 109 subjects with Type 2 diabetes screened for this study are reported in Table 1 (*study 1*). In this cohort, there were 48 Caucasians, 48 African Americans, 4 Hispanics, and 9 Asians. These subjects tended to be middle-aged and obese with elevated random glucose levels and hemoglobin-A1c (HbA1c). Notably, the average plasma vitamin C level in this diabetic cohort was strikingly low at 36 ± 2 μM [vitamin C levels in healthy individuals consuming at least the recommended dietary allowance of 75–90 mg/day are expected to be >50 μM (43, 45)]. Thirty-seven of the 109 subjects whose plasma vitamin C levels were <40 μM were enrolled for further study. Among these 37 subjects, 32 completed the entire study (*study 2*). Five subjects failed to complete the entire study due to personal choice, moving out of the area, or family emergencies. Seventeen subjects were randomized to the placebo treatment group, and 15 subjects were in the vitamin C treatment group. Baseline clinical characteristics of subjects in the placebo and vitamin C group were not statistically different (Table 2, $P > 0.10$ for comparison between placebo and vitamin C groups). Moreover, with the exception of vitamin C levels, the clinical characteristics of the 32 subjects who completed *study 2* were similar to those of the 109 subjects who were initially screened in *study 1* (Tables 1 and 2). Notably, the fasting vitamin C levels in the 32 subjects that completed *study 2* were significantly lower than the already low levels observed in the 109 screened subjects from *study 1* ($P < 0.0001$).

Low vitamin C levels in diabetic subjects. In the 109 diabetic subjects screened for *study 1*, the mean plasma vitamin C level was abnormally low (36 ± 2 μM) when compared with levels in the general population (67, 79). When we analyzed our data for possible associations between low vitamin C levels and various clinical parameters, we did not find any significant associations. Plasma levels of vitamin C were similar between obese and nonobese (36 ± 2 vs. 35 ± 3 μM, $P > 0.6$), men and women (33 ± 3 vs. 38 ± 3 μM, $P > 0.2$), Caucasians and African Americans (35 ± 3 vs. 38 ± 3 μM, $P > 0.6$), subjects with and without hypertension (37 ± 4 vs. 36 ± 2 μM, $P > 0.7$), subjects with and without hypercholesterolemia (33 ± 3 vs. 38 ± 3 μM, $P > 0.2$), and subjects with >7% HbA1c compared with subjects with <7% (33 ± 3 vs. 40 ± 3 μM, $P > 0.09$). Furthermore, we did not observe significant correlations between plasma vitamin C levels and random blood glucose concentrations or the duration of diabetes. Likewise, we did not find significant correlations between vitamin C levels and body mass index (BMI), cholesterol, age, triglycerides, or HbA1c. Thus low vitamin C levels observed in our diabetic cohort could not be associated with or explained by any of the clinical parameters measured.

Vitamin C levels before and after therapy with placebo or vitamin C. In the 32 subjects who completed *study 2*, the baseline mean vitamin C level was exceptionally low (Table 3), but there were no significant differences between levels in subjects randomized to placebo or vitamin C treatment groups (21 ± 3 vs. 23 ± 2 μM, $P > 0.8$). As expected, in the placebo group, plasma vitamin C levels did not change significantly after 4-wk placebo treatment (Table 3, $P > 0.7$). In contrast, after subjects had taken daily oral doses of vitamin C for 4 wk, the plasma vitamin C level of subjects treated with vitamin C increased significantly from 23 ± 2 to 48 ± 6 μM ($P < 0.002$). When changes in plasma vitamin C levels pre- and posttherapy (Δ vitamin C) in the placebo group were compared with the changes in the vitamin C group, there was also a significant difference between Δ vitamin C in the placebo group and the vitamin C group ($P < 0.001$). Interestingly, even though vitamin C levels substantially increased in our diabetic subjects with vitamin C treatment, the magnitude of this increase fell short of the full replenishment expected for healthy individuals. Indeed, healthy subjects with similarly low vitamin C levels would be expected to increase their fasting steady-state plasma vitamin C level to >80 μM after 4 wk of vitamin C at 800 mg/day (47, 53). Taken together, these results indicate that oral supplementation with high-dose vitamin C for 4 wk only partially replenishes low vitamin C levels in subjects with Type 2 diabetes.

Effects of placebo or vitamin C treatment on metabolic parameters. To determine whether high-dose oral vitamin C treatment improves insulin sensitivity in diabetic subjects, we performed hyperinsulinemic isoglycemic glucose clamps on each subject before and after 4-wk administration of placebo or

Table 2. *Study 2: baseline clinical characteristics of study subjects who completed full study*

Treatment Group	n	Sex, M/W	Age, yr	Vitamin C, μM	BMI, kg/m^2	Fasting Glucose, mg/dl	Fasting, Insulin μU/ml	HbAlc, %	Duration of Diabetes, yr	BP, mmHg	HR, beats/min	Cholesterol, mg/dl	LDL, mg/dl	HDL, mg/dl	TG, mg/dl
Placebo	17	5/12	47±3	22±3	36±2	159±12	19±7	8.3±0.5	6.8±1.2	138±5/78±2	77±3	196±9	129±8	48±3	163±30
Vitamin C	15	8/7	49±2	23±2	34±1	156±11	14±2	7.5±0.3	4.3±1.1	140±4/77±2	68±5	202±11	122±11	45±3	231±44

Values are means ± SE; *n,* number of subjects.

Table 3. *Study 2: metabolic parameters of subjects before and after therapy with vitamin C or placebo*

	Placebo (n = 17)				Vitamin C (n = 15)				P (ΔPlacebo vs. ΔVitamin C)
	Pre-Tx	Post-Tx	Δ	P	Pre-Tx	Post-Tx	Δ	P	
Vitamin C, μM	21±3	19±3	−1.6±2.4	>0.7	23±2	48±6	24.9±6.2	<0.002	<0.001
Fasting glucose, mg/dl	159±12	147±13	−12±8	>0.1	156±11	155±9	−2±11	>0.8	>0.4
Fasting insulin, μU/ml	19±6.8	16±4.5	−2.5±2.7	>0.3	14±2.1	13±1.4	−1.1±1.3	>0.4	>0.6
SI_{Clamp}, $10^{-4} \times dl \cdot kg^{-1} \cdot min^{-1}/(\mu U/ml)$	2.06±0.29	2.14±0.31	0.09±0.27	>0.7	2.71±0.46	2.71±0.37	0.00±0.22	>0.9	>0.7
QUICKI	0.308±0.008	0.315±0.008	0.008±0.005	>0.1	0.307±0.006	0.308±0.004	0.001±0.005	>0.8	>0.3
HOMA	7.81±3.02	5.28±1.05	−2.53±2.29	>0.2	5.48±0.85	4.78±0.49	−0.70±0.73	>0.3	>0.4

Values are means ± SE; *n,* number of subjects. Tx, therapy; SI_{Clamp}, glucose clamp-derived index of insulin sensitivity; QUICKI, quantitative insulin-sensitivity check index; HOMA, homeostasis model assessment; Δ, Post-Tx − Pre-Tx.

vitamin C. Values for fasting glucose, fasting insulin, SI_{Clamp}, QUICKI, and HOMA before and after treatment with placebo or vitamin C are reported in Table 3. The mean glucose clamp index of insulin sensitivity (SI_{Clamp}), QUICKI, and HOMA determined at baseline (before treatment) in both the placebo group and the vitamin C group were consistent with the insulin resistance characteristic of Type 2 diabetes (33). There was no significant change in SI_{Clamp} when values for pre- and posttreatment were compared for either the placebo or vitamin C groups (Table 3). Moreover, when ΔSI_{Clamp} for placebo was compared with ΔSI_{Clamp} for vitamin C, there was no significant difference. Similarly, there was no significant change in fasting glucose, fasting insulin, QUICKI, or HOMA when pre- and posttreatment values were compared between groups given placebo or vitamin C. Finally, there was no significant difference between changes in the placebo and vitamin C groups for any of these metabolic parameters. These data suggest that partial replenishment of vitamin C levels achieved after high-dose oral vitamin C treatment is not sufficient to improve insulin resistance in subjects with Type 2 diabetes.

Effects of placebo or vitamin C treatment on vascular function. To determine the effects of placebo or vitamin C treatment on vascular function, we evaluated FBF in each subject in response to infusions of ACh, SNP, and insulin at baseline (before treatment) and after 4-wk treatment with either placebo or vitamin C. The ACh dose-response curves for the placebo group after treatment and the vitamin C group before and after treatment were similar [Fig. 1, $P > 0.6$ by multiple ANOVA (MANOVA)] with an approximately twofold maximal increase in FBF. Despite random assignment to treatment groups, the ACh dose-response curves for the placebo and the vitamin C groups before treatment were significantly different (Fig. 1, $P < 0.01$ by MANOVA). However, the maximal percentage changes in FBF were similar in both placebo and vitamin C groups both before and after treatment (Table 4). Thus endothelium-dependent increases in FBF in response to ACh in our diabetic subjects were unchanged by high-dose oral vitamin C treatment for 4 wk.

To assess endothelium-independent vasodilation, we measured FBF in response to SNP. As with ACh, the SNP dose-response curves for the placebo group after treatment and the vitamin C group before and after treatment were similar (Fig. 2, $P > 0.9$ by MANOVA) with an ~1.6-fold maximal increase in FBF. The SNP dose-response curves for the placebo and the vitamin C groups before treatment were significantly different (Fig. 2, $P < 0.01$ by MANOVA). Nevertheless, the maximal percentage changes in FBF in response to SNP were similar in both the placebo and the vitamin C groups both before and after treatment (Table 4). Thus endothelial-independent increases in FBF in our diabetic subjects were unchanged by high-dose oral vitamin C treatment for 4 wk.

In healthy individuals, systemic intravenous infusion of insulin under glucose-clamp conditions results in significant twofold increases in limb blood flow (16, 71). In the present study, we observed that our diabetic subjects had very blunted vasodilator responses to insulin infusion of ~30% during the glucose clamp both before and after treatment with placebo or vitamin C for 4 wk (Fig. 3). Moreover, no significant differences in vasodilator responses to insulin were noted after treatment with vitamin C or placebo (Table 4). Thus our diabetic subjects had severe impairment in their endothelial response to insulin that was not improved by high-dose vitamin C treatment for 4 wk. In addition to measuring FBF, we also calculated FVR in response to ACh, SNP, and insulin in our

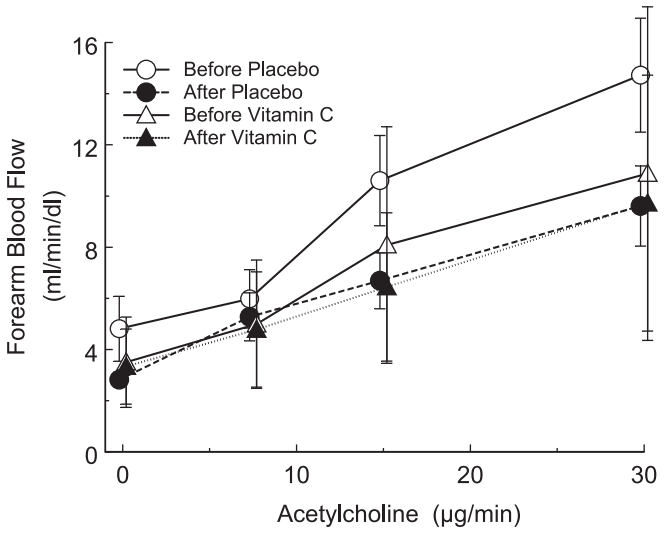

Fig. 1. Endothelium-dependent vasodilation in response to ACh does not significantly change after 4-wk treatment with either placebo or vitamin C. Forearm blood flow (FBF) in response to intra-arterial infusion of increasing doses of ACh was measured by strain-gauge plethysmography in study subjects before and after treatment with placebo or vitamin C for 4 wk as described in METHODS. Data are means ± SE. ACh dose-response curves for subjects before vitamin C treatment, after vitamin C treatment, and after placebo treatment were similar [$P > 0.6$ by multiple ANOVA (MANOVA)]. ACh dose-response curve for subjects before placebo treatment was significantly higher than that of subjects before vitamin C treatment ($P < 0.01$ by MANOVA). Nevertheless, percent change in response to maximal ACh infusion was similar among all 4 groups ($P > 0.6$).

Table 4. *Study 2: maximal percent change over basal in forearm blood flow of subjects in response to ACh, SNP, and insulin before and after therapy with placebo or vitamin C*

	Placebo (n = 17)				Vitamin C (n = 15)				P, (ΔPlacebo vs. ΔVitamin C)
Drug	Pre-Tx	Post-Tx	Δ	P	Pre-Tx	Post-Tx	Δ	P	
ACh	271±46	233±56	−26±57	>0.6	206±31	210±42	2±34	>0.9	>0.6
SNP	180±29	163±23	−16±37	>0.3	195±17	163±23	−34±21	>0.1	>0.6
Insulin	36±10	21±10	−8±14	>0.2	59±25	31±9	−29±25	>0.2	0.02

Values are means ± SE; *n,* number of subjects. SNP, sodium nitroprusside.

study subjects at baseline and after 4-wk administration of placebo or vitamin C. As with FBF, we did not observe significant changes in the FVR response to ACh, SNP, or insulin after subjects were treated with either placebo or vitamin C for 4 wk (data not shown). This was not surprising because mean arterial pressures for our study subjects during infusions of ACh, SNP, and insulin remained essentially constant (data not shown).

Potential effects of variability in vitamin C levels. In the vitamin C treatment group, there were four subjects whose plasma vitamin C levels did not change significantly after 4-wk treatment with vitamin C. This may be the result of physiological differences in these particular subjects or because these subjects failed to take their vitamin C. When queried carefully, all of these subjects claimed to have taken all of their doses of vitamin C. Reanalysis of our data with these four subjects excluded did not significantly change any of our results. Moreover, there was no significant correlation between the magnitude of increase in vitamin C levels and insulin sensitivity in the vitamin C group.

Analysis of blood pressure as a covariate to effects of vitamin C. To determine whether hypertension influences the effects of vitamin C on insulin sensitivity or endothelial function, we analyzed our data using blood pressure as a covariate. We did not observe any significant correlations between blood pressure (mean arterial pressure, systolic blood pressure, or diastolic blood pressure) and insulin sensitivity or endothelial function outcomes after treatment with either vitamin C or placebo (data not shown).

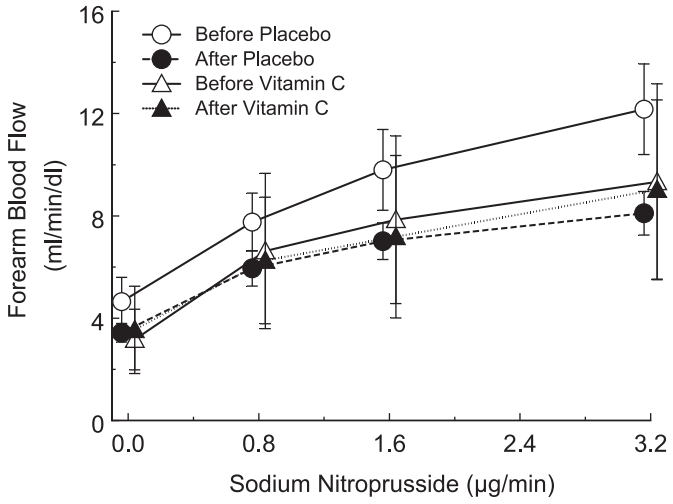

Fig. 2. Endothelium-independent vasodilation in response to sodium nitroprusside (SNP) does not significantly change after 4-wk treatment with either placebo or vitamin C. FBF in response to intra-arterial infusion of increasing doses of SNP before and after treatment with placebo or vitamin C for 4 wk as described in METHODS. Data are means ± SE. SNP dose-response curves for subjects before vitamin C treatment, after vitamin C treatment, and after placebo treatment were similar ($P > 0.9$ by MANOVA). SNP dose-response curve for subjects before placebo treatment was significantly higher than that of subjects before vitamin C treatment ($P < 0.01$ by MANOVA). Nevertheless, percent change in response to maximal SNP infusion was similar among all 4 groups ($P > 0.1$).

DISCUSSION

Vitamin C is an antioxidant whose direct application to endothelium at pharmacological concentrations acutely improves endothelial dysfunction associated with Type 2 diabetes, acute hyperglycemia, or essential hypertension (9, 54, 75). In Type 2 diabetes, reduced serum antioxidant activity correlates with worsened glycemic control (47). Increased oxidative stress and low vitamin C levels are correlated with severity of diabetic neuropathy (83). In a recent review regarding vitamin C levels in Type 2 diabetes, Will and Byers (79) conclude that although people with diabetes may have lower vitamin C levels than normal, various methodological flaws in previous studies introduce uncertainty in vitamin C measurements. Because intra-arterial infusion of vitamin C is beneficial in diabetic subjects who may suffer from low vitamin C levels, full replenishment of vitamin C levels in diabetic subjects may improve their endothelial function. Pharmacological therapies that improve endothelial function also ameliorate insulin resis-

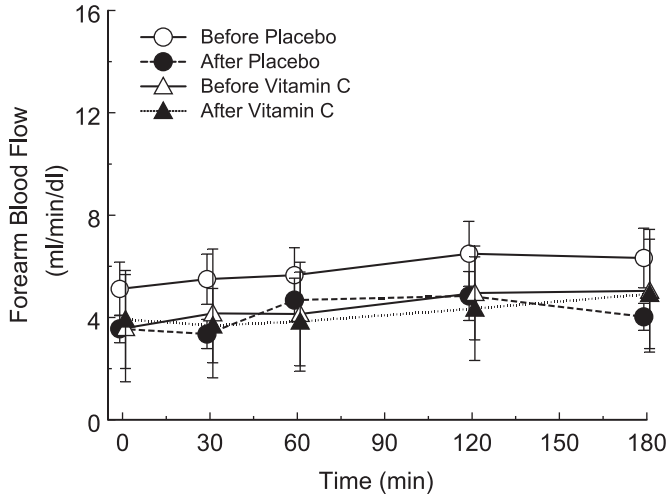

Fig. 3. Systemic intravenous insulin infusion during glucose clamp does not substantially increase FBF in diabetic subjects in placebo or vitamin C group before or after therapy. FBF was measured at various times over a 3-h period by strain-gauge plethysmography in study subjects undergoing a glucose clamp procedure with intravenous insulin infusion of 120 $mU \cdot m^{-2} \cdot min^{-1}$. Data are means ± SE. FBF after 180 min of insulin infusion was modestly higher than that at 0 min in all groups, but there was no statistical difference in time courses of subjects before or after treatment with vitamin C.

tance (36–39, 48). Therefore, we tested whether high dose oral vitamin C supplementation in Type 2 diabetes would improve endothelial function and insulin sensitivity.

Using a state-of-the-art coulometric electrochemical assay, we found that mean vitamin C levels in 109 subjects with Type 2 diabetes screened for our study were unequivocally low (36 ± 2 μM) consistent with previous studies (10, 67, 79). Vitamin C levels were not correlated with any of the other parameters we measured. Low vitamin C levels may be a consequence of diabetes because cellular uptake of vitamin C is regulated by glucose and insulin (11, 18, 69). Diabetic subjects may also have a higher turnover of vitamin C due to increased oxidative stress and oxidation of ascorbate to dehydroascorbic acid in mitochondria (66, 68). Patients with diabetic nephropathy have increased renal clearance of vitamin C (30). It would be of interest to know the oxidation status of our subjects before and after treatment with vitamin C. However, no reliable biomarker of in vivo oxidation has been validated for ascorbic acid or any other antioxidant (28, 57). F_2-isoprostanes are among the best potential biomarkers for lipid oxidation in humans (52). However, in diabetic subjects, ascorbic acid at higher doses than we used in our study has no effect on F_2-isoprostanes (19).

The 32 subjects who completed our intervention study had extremely low mean plasma vitamin C levels (22 μM) and were chosen to maximize the likelihood that vitamin C supplementation would be beneficial. In contrast with the placebo group, we observed a substantial increase in mean plasma vitamin C levels to 48 μM after 4-wk oral therapy (800 mg/day). Strikingly, this incomplete replenishment fell far short of the increase to 80 μM expected for vitamin C-depleted healthy subjects on a similar dose of vitamin C (43–45). Although our subjects did not have overt nephropathy, it is possible that increased renal clearance of vitamin C may account for low vitamin C levels as well as for incomplete replenishment. Indeed, a previous study has documented low vitamin C levels in diabetic subjects despite questionnaire evidence that dietary vitamin C consumption was adequate (67). Thus low vitamin C levels in our subjects may be a consequence of diabetes itself rather than an inadequate intake of vitamin C.

We failed to find any beneficial effects of high-dose oral vitamin C therapy to improve endothelial function or insulin sensitivity in Type 2 diabetes. Our study is the first to report a lack of efficacy of oral vitamin C to improve insulin-mediated vasodilation in resistance arterioles. Our results are consistent with a previous study that did not observe significant differences in fasting glucose, HbA1c, cholesterol, or triglycerides in a double-blind crossover study with 50 diabetic patients taking vitamin C (500 mg/day) or placebo, each for 4 mo (12). Our results are discordant with another study by Paolisso et al. (60) that reported beneficial effects of oral vitamin C (1,000 mg/day for 4 mo) on glucose and lipid metabolism, and free radicals in subjects with Type 2 diabetes. Differences between that study and our study that may account for discordant outcomes include the mean age of the subjects (72 years old), as well as the longer duration and higher dose of vitamin C therapy. It is important to note that Paolisso et al. (60) conducted glucose clamps in only half of their subjects and that mean vitamin C levels after therapy increased to 76 μM. In our study, we used an isoglycemic glucose clamp where blood glucose is maintained at the fasting steady state. This contrasts to euglycemic glucose clamps where blood glucose is maintained in the normal range. We chose the isoglycemic method to avoid confounding effects of acutely lowering blood glucose.

There are several potential reasons why our study was unable to document any beneficial effects of oral vitamin C therapy in Type 2 diabetes. One possibility is that our intervention duration and dose of vitamin C were inadequate. Steinberg (70) has hypothesized that antioxidant therapy may take more than 5 yr to demonstrate any benefit because the primary mechanism of these agents may be preventative. However, this reasoning may not apply to our study because direct administration of vitamin C to vascular endothelium is sufficient to acutely reverse endothelial dysfunction in diabetes (75), and other therapies that improve endothelial function improve insulin sensitivity within 1 mo (39, 48, 58). On the other hand, acute local improvement in forearm endothelial function in hypertensive subjects did not improve insulin-mediated glucose uptake in the treated forearm (54). However, mechanisms of endothelial dysfunction in hypertension and diabetes may differ.

It is unlikely that a higher dose of vitamin C would substantially increase levels of vitamin C in our subjects. The daily dose of 800 mg chosen in our study is a high and safe dose based on studies of depletion-replenishment of vitamin C in healthy subjects (44). In healthy subjects, vitamin C levels in plasma reach a maximum of ~80 μM at oral vitamin C doses of 400 mg/day and do not increase even at doses up to 2,500 mg/day (45). Thus the dose used in our study is twice that required to saturate plasma levels of vitamin C in healthy subjects and is much higher than the recommended dietary allowance (17, 45). It is also unlikely that our study is underpowered because our sample size is adequate to detect a 10% change in endothelial function and insulin sensitivity with more than 90% power. It seems more likely that a failure to achieve full replenishment of vitamin C levels accounts for our negative results. This may be due to a specific defect in vitamin C handling, because a recent study showed that oral antioxidant therapy with vitamin C and E combined was able to improve endothelial function only in Type 1 but not Type 2 diabetes (10). These results suggest that genetic contributions to insulin resistance play a role in the abnormal vitamin C handling observed in Type 2 diabetes. Another possible factor contributing to our negative results is that endothelial cells make oxygen radicals instead of eliminating them (27). Thus vitamin C studies may only work in the short term (62) because the ability to regenerate tetrahydrobiopterin may be time limited.

Improving bioavailability of NO is one mechanism that may improve endothelial function in Type 2 diabetes (56). SOD and vitamin C prevent the rapid inactivation of NO by superoxide anion (26). Interestingly, the concentration of vitamin C required to preserve NO-mediated arterial relaxation is higher than the concentration required to scavenge superoxide (32). However, high concentrations of vitamin C (>80 μM) are required to preserve NO-dependent endothelial function because vitamin C only competes with NO for superoxide anion at these high concentrations (32, 65). Thus incomplete replenishment of vitamin C levels achieved in our subjects is the likely explanation for a lack of improvement in endothelial function and insulin sensitivity after high-dose oral vitamin C

therapy. Oxidized ascorbate (dehydroascorbic acid) may enter endothelial cells through glucose transporters. Thus, in diabetic subjects, dehydroascorbic acid transport may be inhibited by high glucose concentrations (25, 63), and intracellular ascorbate concentrations may have remained low despite supplementation. It remains possible that full replenishment of vitamin C levels in subjects with Type 2 diabetes may have beneficial effects to improve both endothelial dysfunction and insulin resistance. It is also possible that only high levels of vitamin C applied directly to the endothelium (not achievable by oral dosing) are sufficient to improve diabetic endothelial dysfunction. Understanding why high-dose oral vitamin C does not fully replenish patients with Type 2 diabetes is an important goal for future studies, as well as pharmacokinetic studies of vitamin C in patients with Type 2 diabetes to determine whether a renal leak may explain the incomplete replenishment of vitamin C that we observed.

GRANTS

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