Ascorbate Restores Endothelium-Dependent Vasodilation Impaired by Acute Hyperglycemia in Humans

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Background—Endothelium-dependent vasodilation is impaired in patients with insulin-dependent and non–insulin-dependent diabetes mellitus and restored by vitamin C administration, implicating a causative role for oxidant stress. Hyperglycemia per se attenuates endothelium-dependent vasodilation in healthy subjects. Accordingly, this study investigated whether impaired endothelium-dependent vasodilation caused by hyperglycemia in nondiabetic humans is restored by administration of the antioxidant vitamin C.

Methods and Results—Endothelium-dependent vasodilation was measured by incremental brachial artery administration of methacholine chloride (0.3 to 10 μg/min) during euglycemia, after 6 hours of hyperglycemia (300 mg/dL) created by dextrose (50%) intra-arterial infusion, and with coadministration of vitamin C (24 mg/min) during hyperglycemia. Endothelium-dependent vasodilation was significantly diminished by hyperglycemia ($P=0.02$ by ANOVA) and restored by vitamin C ($P=0.04$). In contrast, endothelium-dependent vasodilation was not affected by equimolar infusions of mannitol, with and without vitamin C coinfusion ($P=$NS). Endothelium-independent vasodilation was measured by incremental infusion of verapamil chloride (10 to 300 μg/min) without and with coadministration of N^G-monomethyl-L-arginine (L-NMMA). In the absence of L-NMMA, endothelium-independent vasodilation was not significantly altered during hyperglycemia ($P=$NS) but was augmented by vitamin C ($P=0.04$). The coadministration of L-NMMA eliminated the vitamin C–related augmentation in verapamil-mediated vasodilation.

Conclusions—Vitamin C administration restores endothelium-dependent vasodilation impaired by acute hyperglycemia in healthy humans in vivo. These findings suggest that hyperglycemia may contribute in part to impaired vascular function through production of superoxide anion. **(*Circulation*. 2001;103:1618-1623.)**

Key Words: nitric oxide ■ diabetes mellitus ■ antioxidants ■ glucose

Atherosclerosis is the leading cause of morbidity and mortality in patients with diabetes mellitus. One putative cause of atherosclerosis in diabetes mellitus is impaired endothelial function stemming from reduced bioavailability of nitric oxide. Endothelium-derived nitric oxide regulates vasomotor tone and performs many important vasoprotective functions such as inhibiting platelet aggregation and preventing adhesion of inflammatory cells (leukocytes) to the endothelial surface.[1] Endothelium-dependent vasodilation is impaired in animal models and humans with type 1 and type 2 diabetes mellitus.[2–4] Administration of vitamin C, an antioxidant, improves endothelium-dependent vasodilation in patients with either type of diabetes mellitus,[3,4] thus implicating inactivation of nitric oxide by oxygen-derived free radicals as a mechanism of endothelial dysfunction in both forms of diabetes.[5,6]

Hyperglycemia may be a fundamental abnormality underlying the mechanism that causes endothelial dysfunction in diabetes. Indeed, endothelium-dependent relaxation of aortic rings from healthy rabbits are impaired when incubated in a hyperglycemic milieu.[7] Our laboratory and others have demonstrated that endothelium-dependent vasodilation is impaired in healthy subjects after 6 hours of a hyperglycemic clamp.[8,9] Moreover, increases in blood glucose further depress endothelium-dependent vasodilation in subjects with type 2 diabetes mellitus.[10] Taken together, these findings raise the possibility that endothelial dysfunction in diabetes may occur as a result of oxidant stress induced by hyperglycemia. Hyperglycemia may promote superoxide production as a consequence of glucose auto-oxidation, the formation of advanced glycation end products, abnormal arachidonic acid metabolism and its coupling to cyclo-oxygenase catalysis, by activating protein kinase C, by depleting tetrahydrobiopterin, and by increasing the activity of nitric oxide synthase.[11–17]

Therefore, the purpose of this study was to test the hypothesis that hyperglycemia per se impairs endothelium-dependent vasodilation in humans by inducing the formation of superoxide anion and reducing the bioavailability of endothelium-derived nitric oxide. To test this hypothesis, we sought to determine whether administration of the antioxidant

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vitamin C would improve the impaired endothelium-dependent vasodilation caused by experimental hyperglycemia in vivo in healthy, nondiabetic humans.

Methods

The protocol was approved by the Human Research Committee of Brigham and Women's Hospital. Twenty-eight healthy volunteers were recruited by newspaper advertisement and provided written informed consent. All subjects underwent screening history, physical examination, and laboratory analysis, including complete blood count, serum electrolytes, fasting glucose, blood urea nitrogen, creatinine, transaminases, alkaline phosphatase, and a lipid profile. Subjects with hypertension, history of tobacco use, LDL cholesterol or total cholesterol greater than the 75th percentile for age and sex, cardiovascular disease, or other disease were excluded.

Subjects were studied in the morning in the postabsorptive state, fasting after the previous midnight. Cyclo-oxygenase inhibitors, alcohol, and caffeine were prohibited for 12 hours before study initiation. With the use of subcutaneous lidocaine anesthesia and sterile conditions, a 20-gauge Teflon catheter was inserted into the brachial artery of the nondominant forearm for drug infusion and blood pressure measurement. Intravenous cannulas were inserted into antecubital veins of each arm. The vascular research laboratory was quiet, dimly lit, and temperature controlled at 23°C. Subjects rested for a minimum of 30 minutes after insertion of the catheters before baseline hemodynamic data were acquired.

Forearm Hyperglycemic Clamp Method

A forearm hyperglycemic clamp was used to raise and maintain forearm glucose concentration at 300 mg/dL (16.7 mmol/L) as previously described.[8] A 50% dextrose solution was infused into the forearm through the brachial artery catheter. Fifteen minutes after the infusion was started, ipsilateral antecubital venous blood was obtained, the blood glucose level was determined, and the infusion rate was adjusted. The infusion rate was adjusted every 10 to 15 minutes for the duration of the study to maintain the hyperglycemic clamp at 300 mg/dL. In addition, the somatostatin analog octreotide was infused at 30 ng $\cdot$ kg^{-1} $\cdot$ min^{-1} to suppress pancreatic insulin because insulin is a known vasodilator[18,19] whose vascular effects are mediated at least in part by endothelium-derived nitric oxide. The octreotide infusion was initiated 30 minutes before the first hemodynamic measurement and maintained throughout each protocol. No vasoactive effects have been identified in studies that used the same doses of octreotide.[20] Systemic glucose and insulin samples were obtained at baseline, 3 hours into the clamp, and after 6 hours of hyperglycemic clamp from the contralateral antecubital vein.

Hemodynamic Measurements

Bilateral forearm blood flow was measured by venous-occlusion, mercury-in-silastic, strain-gauge plethysmography, by established methods.[21] During data acquisition, wrist cuffs were inflated to 200 mm Hg to exclude the hand circulation. A venous occlusion pressure of 40 mm Hg was generated by cuffs placed on each arm above the elbow for each measurement of blood flow, which is reported as mL/100 mL of tissue per minute. Arterial blood pressure was measured by the brachial artery cannula. The cannula was attached to a pressure transducer contiguous with an amplifier on a Gould physiological recorder. Heart rate was determined by the R-R interval of a continuous ECG monitor.

Laboratory Analyses

Whole-blood glucose concentration was measured at the bedside by means of the glucose oxidase method, with a glucose reflectometer. Reported values represent analyses performed subsequently on plasma with a Glucose Analyzer II (Beckman Instruments Inc). Insulin was measured with a radioimmunoassay. Osmolality was determined by freezing point depression. All sample measurements were performed in duplicate.

Experimental Protocols

The effects of 6 hours of hyperglycemia and the acute administration of vitamin C on endothelium-dependent vasodilation were investigated in 18 healthy subjects. First, during fasting euglycemia, basal forearm blood flow and the blood flow response to 4-minute intra-arterial infusions of incremental doses of methacholine chloride (0.3, 1.0, 3.0, and 10.0 μg/min) were assessed to determine vasodilation in response to endothelium-derived nitric oxide. Forearm glucose concentration was then clamped at 300 mg/dL (16.7 mmol/L) by intra-arterial infusion of 50% dextrose for 6 hours, as described above. After 6 hours of hyperglycemic clamp, a time frame based on our previous experience,[8] basal forearm blood flow, and the blood flow responses to methacholine were measured. After discontinuation of methacholine and reestablishment of basal flow, vitamin C was infused intra-arterially at a dose of 24 mg/min in conjunction with the hyperglycemic clamp. Ten minutes thereafter, basal forearm blood flow and methacholine-induced increases in forearm blood flow were measured again.

As a time and osmolality control, the protocol was repeated in 10 subjects in whom dextrose was replaced with an equimolar 25% mannitol infusion to maintain a hyperosmolar clamp. All of these subjects previously participated in a hyperglycemic clamp. The dextrose infusion rates in the first study were used as a guide for mannitol infusion rates. Venous samples from the study arm were obtained to record the osmolality attained. Methacholine dose-response curves were measured before and after 6 hours of the hyperosmolar clamp. The dose response to methacholine during the hyperosmolar clamp was then measured during coinfusion of vitamin C as described above. Mannitol has modest antioxidant properties as a hydroxyl radical scavenger but does not scavenge other oxidants including superoxide anion and lipid peroxides.[22]

To ascertain whether the vascular effects of hyperglycemia and vitamin C were limited to the endothelium, a subset of 9 subjects was studied on a separate occasion with the calcium channel blocker verapamil at doses of 10, 30, 100, and 300 μg/min. Forearm blood blow measurements were made under basal conditions and with verapamil infusions during euglycemia, after 6 hours of hyperglycemic clamp, and during hyperglycemic clamp along with vitamin C administration. Verapamil causes vasorelaxation by a direct action on vascular smooth muscle. However, the resultant increase in blood flow may induce release of nitric oxide from nitric oxide synthase.[23–26] To eliminate the contribution of nitric oxide synthase from the vasodilator effect of verapamil, N^G-monomethyl-L-arginine (L-NMMA) was coinfused at 2 mg/min with verapamil in 6 additional subjects during the hyperglycemic clamp. Forearm blood flow responses to verapamil were made in the subjects before and after vitamin C administration.

Statistical Analyses

Values are reported as mean$\pm$SEM. Basal forearm blood flow, osmolality, glucose concentration, and insulin concentration were compared by paired 2-tailed t tests. Statistical analyses of the dose-response curves for each drug (methacholine and verapamil) were conducted by the absolute increase in blood flow from the resting flow rate. Two-way repeated-measures ANOVA was performed to compare the dose-response curves during euglycemic conditions and after 6 hours of hyperglycemic clamp and the dose-response curves during the hyperglycemic clamp before and after the coinfusion of vitamin C. Statistical significance was accepted at the 95% confidence level ($P \leq 0.05$).

Results

Baseline Characteristics

Twenty-eight healthy subjects, including 10 men and 18 women (age, 26.6$\pm$4 years), participated in the protocols. Mean blood pressure was 116/66$\pm$13/7 mm Hg, fasting glucose was 71$\pm$11 mg/dL, baseline insulin level was 1.1$\pm$0.5 mU/mL, and total cholesterol concentration was

TABLE 1. Basal Forearm Blood Flow at Baseline, During Clamp, and With Vitamin C Administration

	Protocol		
Protocol Stage	Methacholine	Mannitol	Verapamil
Baseline euglycemia	2.3±0.2	1.9±0.1	1.6±0.1
6-h hyperglycemic or hyperosmolar clamp	3.3±0.4*	3.5±0.4*	2.6±0.4†
Hyperglycemic clamp+vitamin C	4.7±0.6*	4.7±0.4*	4.4±0.4‡

All measurements are forearm blood flow (mL/100 mL tissue±SEM) and comparisons of resting flow made to previous conditions.

*$P<0.01$.

†$P=0.052$.

‡$P=0.02$.

166±30 mg/dL. Serum cholesterol, insulin, glucose, and mean arterial pressure were within normal limits in all subjects.

Effect of Hyperglycemia, Hyperosmolality, and Vitamin C on Basal Forearm Blood Flow

Basal, that is, resting, forearm blood flow was measured in all conditions (Table 1). Resting forearm blood flow in the experimental forearm increased from 2.3±0.2 mL/100 mL per minute during euglycemia to 3.3±0.4 mL/100 mL per minute during hyperglycemia ($P<0.01$) and increased further to 4.7±0.7 after vitamin C administration ($P<0.01$). Resting forearm blood flow increased also during the hyperosmolar clamp from 1.9±0.1 to 3.5±0.4 mL/100 mL per minute during ($P<0.01$) and then to 4.7±0.4 mL/100 mL per minute ($P<0.01$) with vitamin C administration (Table 1). The pattern of increase to hyperglycemia and subsequently with vitamin C was also observed in the verapamil experiments.

Effect of Hyperglycemia and Vitamin C on Response to Methacholine

During euglycemia, the ipsilateral forearm venous glucose concentration was 71±11 mg/dL. Forearm glucose averaged 379±100 mg/dL over the 6 hours of hyperglycemic clamp, whereas the systemic (contralateral venous) concentration averaged 121±32 mg/dL. Systemic insulin levels increased only slightly, from 1.06±0.5 to 2.82±1.1 μU/mL ($P<0.01$). Incremental doses of methacholine increased forearm blood flow during both euglycemia and hyperglycemia. However, compared with euglycemia, the forearm blood flow response to intra-arterial methacholine was reduced significantly after the 6-hour hyperglycemic clamp. (Figure 1, $P=0.02$). Thereafter, the administration of vitamin C significantly increased the forearm blood flow response to methacholine compared with that during hyperglycemia alone (Figure 1, $P=0.04$), achieving a response similar to that observed during euglycemia. Heart rate, mean arterial pressure, and the contralateral forearm blood flow were not significantly affected by methacholine infusion, hyperglycemia, or vitamin C administration.

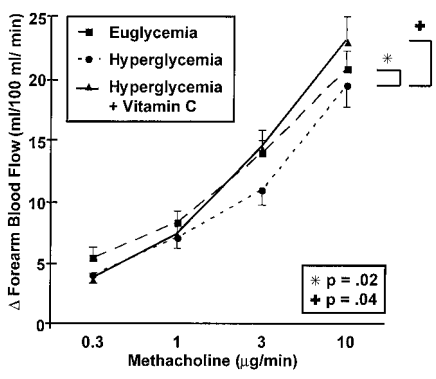

Figure 1. Effect of hyperglycemia and vitamin C on endothelium-dependent vasodilation. Increase in forearm blood flow from baseline induced by methacholine at baseline euglycemia, during hyperglycemic clamping, and coadministration of vitamin C during hyperglycemia. Endothelium-dependent vasodilation was significantly attenuated during hyperglycemia ($P=0.02$) and restored by vitamin C administration ($P=0.04$).

TABLE 2. Baseline Parameters Before and During Clamping

	Hyperglycemia	Mannitol
Baseline glucose, mg/dL	71±11	76±12
6-h glucose, mg/dL	379±100	97±10
Baseline osmolality	278±5	282±5
6-h osmolality	296±3	307±17

All measurements are mean±SEM.

Effect of Hyperosmolality and Vitamin C on Response to Methacholine

Forearm osmolality was clamped at 300±8 mOsm/kg during the 6-hour hyperosmolar clamp. Baseline and 6-hour glucose and osmolality are reported in Table 2. Incremental infusions of methacholine increased forearm blood flow in a dose-dependent manner during normal and hyperosmolar conditions. In contrast to the hyperglycemic clamp, the forearm blood flow response to methacholine did not change significantly after 6 hours of hyperosmolality (Figure 2). Moreover, the administration of vitamin C during the hyperosmolar clamp did not alter the blood flow response to methacholine (Figure 2). Neither serum glucose nor insulin levels were affected by the mannitol infusion.

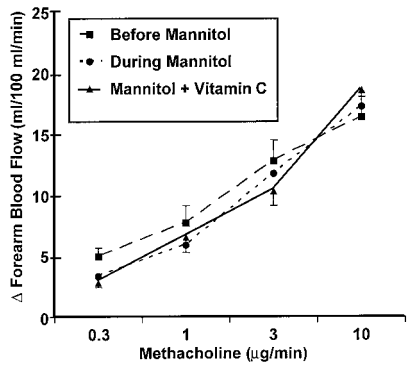

Figure 2. Effect of hyperosmolality and vitamin C on response to methacholine. Increase in forearm blood flow from baseline induced by methacholine at baseline, during hyperosmolar clamping, and coadministration of vitamin C during hyperosmolality. No significant difference was detected in response to increase in osmolality nor addition of vitamin C (P=NS).

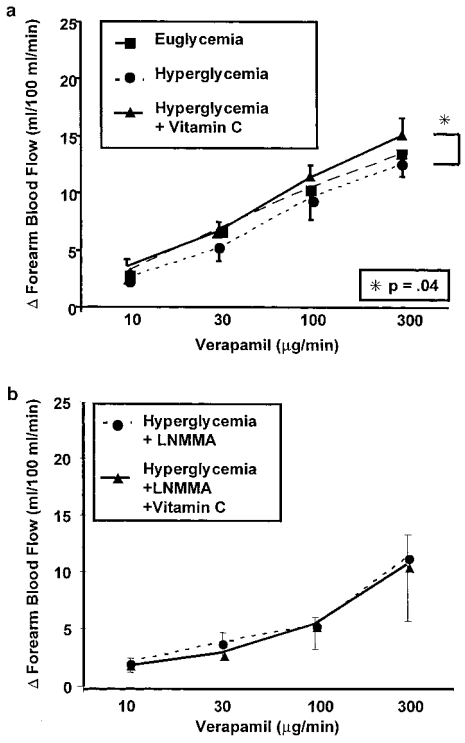

Figure 3. a, Effect of hyperglycemia and vitamin C on endothelium-independent vasodilation. Increase in forearm blood flow from baseline induced by verapamil at baseline euglycemia, during hyperglycemic clamping, and coadministration of vitamin C during hyperglycemia. Verapamil-mediated vasodilation was not different during hyperglycemia (P=NS) but was augmented by vitamin C administration (P=0.04). b, Effect of L-NMMA on response to verapamil and vitamin C. Increase in forearm blood flow from baseline induced by verapamil during hyperglycemic clamping and coinfusion of L-NMMA and during L-NMMA and vitamin C coadministration during hyperglycemia. There was no significant effect of vitamin C on response to verapamil during coinfusion of L-NMMA (P=NS).

Effect of Hyperglycemia and Vitamin C on Response to Verapamil

The response to verapamil was assessed before and after forearm glucose was clamped at 353±74 mg/dL over 6 hours. The infusion of verapamil increased forearm blood flow in a dose-dependent manner during euglycemia and hyperglycemia, and the dose response was not significantly different between euglycemic and hyperglycemic conditions. The infusion of vitamin C, however, did enhance the forearm blood flow response to verapamil (P=0.04) (Figure 3a). Six subjects underwent the second verapamil protocol, in which measurements were made during coinfusion of L-NMMA. In these subjects, administration of vitamin C did not change the forearm blood flow response to verapamil (Figure 3b).

Discussion

The important and novel finding of this study is that vitamin C improved the abnormality in endothelium-dependent vasodilation that was caused by experimental hyperglycemia in healthy, nondiabetic human subjects in vivo. This observation suggests that hyperglycemia, by increasing the production of oxygen-derived free radicals, decreases the bioavailability of nitric oxide. Oxygen-derived free radicals rapidly combine with nitric oxide, decrease its bioavailability, and thereby impair normal endothelial function.[5,6]

Impaired endothelium-dependent vasodilation and, by extension, decreased bioavailability of endothelium-derived nitric oxide, has been widely demonstrated in animal models of diabetes mellitus and in patients with both type 1 and type 2 diabetes.[2,21,27–29] Augmented generation of oxygen-derived free radicals as a cause of this endothelial dysfunction has been implicated by studies in humans with type 1 and type 2 diabetes mellitus in whom infusions of the antioxidant vitamin C restores endothelium-dependent vasodilation toward normal.[3,4] One feature common to the in vitro, animal, and human models of diabetes is hyperglycemia. Hyperglycemia per se has been shown to attenuate endothelium-dependent vasodilation in healthy, nondiabetic animal and human arteries.[8,30,31]

Hyperglycemia and Oxidant Stress

Elevations of F_2-isoprostane, a marker of oxidant stress, have been found in subjects with both types of diabetes.[32] Improved glycemic control decreases the level of F_2-isoprostane, suggesting a causal relation between blood glucose levels and oxidant stress.[32] There are several mechanisms by which hyperglycemia may increase oxidant stress,[33] including condensation of excess glucose with plasma proteins to form advanced glycation end products and superoxide anion; glucose auto-oxidation; abnormal arachidonic acid metabolism; activation of protein kinase C; depletion of a cofactor for nitric oxide synthase, tetrahydrobiopterin; and activation of the aldose reductase pathway.[11–16]

Recent work has suggested that nitric oxide synthase may be an important source for superoxide in hyperglycemia.[17] Cosentino and colleagues[17] examined the effect of exposing endothelial cells in vitro to 22 mmol/L glucose. Production of nitric oxide and superoxide was compared between human endothelial cell cultures exposed to 5 mmol/L glucose and 22 mmol/L glucose. The group demonstrated that endothelial exposure to hyperglycemia caused nitric oxide synthase to modestly increase its production of nitric oxide by 40% and markedly increase its production of superoxide anion by 300%.

Our findings support an important role for superoxide anion as a cause of abnormal endothelium-dependent vasodilation caused by hyperglycemia. Indeed, the reduction in forearm blood flow response to hyperglycemia was reversed when vitamin C was infused during the hyperglycemic clamp. These findings cannot be attributed to the hyperosmolar effects of hyperglycemia because the forearm dose response to methacholine was not affected by hyperosmolality alone or with concurrent vitamin C.

Hyperglycemia did not change the forearm blood flow response to verapamil, confirming previous observations[8]; however, the blood flow response to verapamil was increased during hyperglycemia when vitamin C was administered. L-NMMA eliminated the increase in flow seen during coinfusion of vitamin C with verapamil. There are two potential explanations for these observations. First, the verapamil infusion caused release of nitric oxide as a consequence of the

increase in flow and resultant greater bioavailability of nitric oxide. L-NMMA eliminated this potential endothelium-dependent component of verapamil-mediated vasodilation; therefore, the scavenging of superoxide anion by vitamin C did not augment flow.

Alternatively, hyperglycemia may act independent of flow to alter the function of nitric oxide synthase, augmenting production of both superoxide anion and nitric oxide but preferentially producing a greater proportion of superoxide anion.[17] Hyperglycemia has been demonstrated to affect the activity of nitric oxide synthase by depleting its cofactor, tetrahydrobiopterin, and by activating protein kinase C.[12,14,16,34] If this were the case, vitamin C would reveal increased ambient nitric oxide by scavenging the increased superoxide and augment vasodilation, as was observed in this study when vitamin C was coinfused with verapamil. Indeed, this reasoning is supported by our experiments with L-NMMA, which inhibits the production of nitric oxide by endothelial nitric oxide synthase.[35,36] Thus, L-NMMA, by inhibiting the hyperglycemia-mediated increased production of nitric oxide by nitric oxide synthase, abrogated the improvement in blood flow that occurred when vitamin C was coinfused with verapamil.

Effect of Hyperglycemia on Basal Forearm Blood Flow

Even though hyperglycemia decreased endothelium-dependent vasorelaxation, basal forearm blood flow increased after the 6-hour hyperglycemic clamp. It is likely that the increase in basal flow was largely an effect of osmolality because basal forearm flow also increased during the hyperosmolar clamp. The increase in basal forearm blood flow that accompanied the vitamin C infusion occurred in both settings, hyperglycemia and hyperosmolality, suggesting that this was either a time-dependent phenomenon or a consequence of decreased inactivation of ambient nitric oxide.

Antioxidant Properties of Vitamin C

In these experiments, vitamin C, a water-soluble antioxidant, was used to test the primary hypothesis that hyperglycemia impairs endothelium-dependent vasodilation in humans through production of superoxide anion and consequent inactivation of nitric oxide. Vitamin C may act extracellularly as a superoxide anion scavenger and intracellularly by affecting the redox state. The infusion of 24 mg/min of vitamin C for 10 minutes yields a local forearm concentration of 1 to 10 mmol/L. Jackson et al[37] demonstrated in vitro that this concentration of vitamin C competes effectively with endogenous antioxidants for superoxide anion. Reduced vitamin C also may increase nitric oxide by increasing the activity of nitric oxide synthase directly; however, Heller et al[38] demonstrated in vitro that intracellular ascorbic acid transport was time dependent and did not affect nitric oxide synthase activity at 1 hour. Thus, it is likely that the short-duration, high-dose infusion of vitamin C used in this study acted by scavenging extracellular oxygen-derived free radicals.

Both reduced and oxidized vitamin C inhibit intracellular transport of glucose through the GLUT 1 transporter[39] and conceivably might prevent glucose from impairing endothelium-dependent vasodilation by this mechanism. However, the hyperglycemic clamp had been maintained for 6 hours before vitamin C administration, and there is evidence that the effects of prolonged hyperglycemia remain hours after restitution of normal extracellular glucose concentration.[40]

Conclusions

The results of this investigation indicate that in healthy humans, vitamin C reverses the impairment of endothelium-dependent vasodilation caused by acute hyperglycemia. This observation is consistent with the postulate that vitamin C increases the bioavailability of nitric oxide by scavenging excess oxygen-derived free radicals produced by hyperglycemia. We speculate that an important mechanism whereby hyperglycemia induces oxidant stress is through the stimulation of nitric oxide synthase, preferentially increasing the synthesis of superoxide anion over nitric oxide. Taken together, the findings of this study support a fundamental role of hyperglycemia per se in mediating endothelial dysfunction in patients with diabetes mellitus.

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