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Original Studies

Chronic Vitamin E Administration Improves Brachial Reactivity and Increases Intracellular Magnesium Concentration in Type II Diabetic Patients

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Abstract

Vascular disease accounts for the majority of the clinical complications in diabetes mellitus. As an exaggerated oxidative stress degree has been postulated as the link between diabetes mellitus and endothelial function, a possible positive effect of plasma vitamin E (Vit.E) administration on brachial reactivity could be postulated. Our study aims at investigating the possible effect of chronic Vit.E administration on brachial reactivity, oxidative stress indexes, and intracellular magnesium and calcium content in type II diabetic patients free of diabetic complications. Forty adult, type II diabetic patients were enrolled in the study, which was deigned as a double blind, randomized *vs.* placebo trial. At baseline all patients underwent the following tests: 1) anthropometric and metabolic examinations, 2) evaluation of oxidative stress indexes, 3) intracellular magnesium and calcium measurements, and 4) determination of arterial compliance and distensibility. Then, all patients were randomly assigned to Vit.E treatment at a dose of 600 mg/day (Evion Forte; n = 20) or placebo (n = 20) over 8 weeks. At the end of this treatment period, a complete reevaluation of the patients was made. Vit.E treatment was associated with a significant improvement in the percent change in brachial artery diameter ($P < 0.03$) and oxidative stress indexes ($P < 0.005$). In the Vit.E group, the percent change in brachial artery diameter correlated positively with the percent change in oxidative stress indexes (oxidized/reduced glutathione, Trolox-equivalent antioxidant capacity, thiobarbituric acid reaction products, lipid peroxides) and intracellular cation content (magnesium and calcium). After adjustment for age, sex, body mass index,

and wait/hip ratio, all of these correlations remained significant ($P < 0.03$ for all). Furthermore, adjusting for glycosylated hemoglobin, plasma total cholesterol, and homeostatic model index, brachial artery diameter was still correlated with the percent change in oxidative stress indexes ($P < 0.04$ for all). Nevertheless, the relationship between the percent change in brachial artery diameter and oxidative stress indexes was no longer significant after adjustment for intracellular Mg and Ca^{2+}. In conclusion, our study demonstrates that chronic administration of Vit.E improves brachial artery reactivity in patients with type II diabetes mellitus. Such an effect seems mediated by a reduction in oxidative stress and a regulation of intracellular calcium and magnesium contents.

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THE OCCURRENCE of vascular disease accounts for the majority of the clinical complications in diabetes mellitus (1). Previous studies have demonstrated an exaggerated oxidative stress degree in type II diabetic patients (2); this condition leads to an inactivation of nitric oxide (NO), thus resulting in endothelial dysfunction (3). The evaluation of flow-mediated vasodilatation of the brachial artery has been previously suggested as an index of endothelial dysfunction and early atherosclerosis (4). Antioxidant agents have been found to be useful in improving endothelial function, via a reduction of oxidative stress degree, in animal models of hypercholesterolemia (5) and diabetes (6). However, only the effect of acute intraarterial vitamin C (Vit.C) administration has been evaluated in diabetic patients (7, 8).

Vitamin E (Vit.E) is a potent antioxidant acting as scavenger of superoxide and other reactive species known to be decreased in diabetic tissue and blood (9). A possible positive effect of plasma Vit.E on brachial reactivity could be postulated. Furthermore, increased oxygen free radical production lowers the intracellular magnesium concentration (10), and this condition leads to an altered intracellular calcium content, resulting in vasoconstriction (11). In light of such evidence, Vit.E administration might also regulate the intracellular magnesium concentration.

Our study aims at investigating the possible effect of chronic Vit.E administration on brachial reactivity, oxidative stress indexes, and intracellular magnesium and calcium contents in type II diabetic patients free of diabetic complications.

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Patients

Forty adult, type II diabetic patients were enrolled in the study. The mean duration of diabetes was 7.8 yr. All patients were free from micro- and macroangiopathy (evaluated by routine laboratory analyses, fundus oculi, ergometric test, and ultrasound examination), had normal renal function (microalbuminuria, <20 μg/24 h; plasma creatinine, <100 μmol/L), and the presence of diabetic neuropathy was excluded by Ewing's tests (12). All patients were treated by diet and glibenclamide (Gliben Abiogen Pharma, Pisa, Italy; 5 mg twice daily) and had good metabolic control as demonstrated by hemoglobin A_{1c} levels. Neither diet nor glibenclamide treatment was discontinued or changed in dosage throughout the study. More detailed characteristics of the patients are given in Table 1.

View this table: [in this window] [in a new window]	**Table 1.** Clinical characteristics of the study groups

The study was approved by the ethical committee of our institution after written consent was obtained from each patient participating in the study.

Study design

The study was designed as a double blind, randomized *vs.* placebo trial. At baseline all patients underwent the following tests: 1) anthropometric and metabolic examinations, 2) evaluation of oxidative stress indexes, 3) intracellular magnesium and calcium measurements, and 4) determination of arterial compliance and distensibility. Then, all patients were randomly assigned to Vit.E treatment at dose of 600 mg/day (Evion Forte, Bracco, Italy; n = 20) or placebo (n = 20). Each treatment lasted 8 weeks. At the end of this treatment period, a complete reevaluation of the patients was made.

Anthropometric and metabolic examinations. Weight and height were measured by standard technique. Body fat and fat-free mass were measured using a four-terminal bioimpedance analyzer (RJL Spectrum Bioelectrical Impedance, BIA 101/SC Akern, RJL-System, Florence, Italy) (13). Body mass index was calculated as body weight divided by height squared. Waist circumference was measured at the midpoint between the lower rib margin and the iliac crest (normally the umbilical level), and hip circumference was measured at the trochanter level. Both circumferences were measured at the nearest 0.5 cm with plastic tape, and the ratio between them provided the waist/hip ratio.

Insulin sensitivity was derived by homeostatic model assessment (HOMA) according to Matthews *et al.* (14). All tests were performed in the morning after an overnight fast (12 h at least).

Evaluation of oxidative stress indexes. The degree of oxidative stress was evaluated through determination of thiobarbituric acid reaction products (TBARS), oxidized/reduced glutathione ratio (GSH/GSSG), and hydrolipoperoxides (LPO) as reported previously (15). In addition, the total plasma antioxidant capacity was assessed as Trolox equivalent antioxidant capacity (TEAC), according to the method of Pellegrini *et al.* (16).

Intracellular magnesium and calcium measurements. Blood samples for intracellular magnesium (Mgi) and calcium (Ca^{2+}) measurements were collected into tubes containing heparin. Intracellular Mgi and Ca^{2+} were determined as previously reported (17). Briefly, erythrocytes were isolated by centrifugation (5000 rpm for 15 min), and the precipitate was washed three times with an isotonic saline solution (150 mmol/L NaCl). Subsequently, cells were incubated for 90 min in Krebs-Ringer buffer of the following composition: 2.5 mmol/L $NaCl_2$, 1.2 mmol/L Mg/Cl_2, and 20 mmol/L $NaHCO_3$. Solutions were continuously gassed with a mixture of 95% O_2 and 5% CO to a pH of 7.4, and the temperature was kept at 37 C. Cells were counted to normalize samples, then were lysed osmotically by the addition of deionized water, allowing the solution to stand for 30 min. Thus, the solution was centrifuged, and the supernatant was kept at -20 C until magnesium and calcium determinations were made by atomic absorption spectrophotometry using a Perkin-Elmer Corp. apparatus (Perkin-Elmer Corp., Norwalk, CT). All assays were performed in duplicate.

Brachial reactivity. Brachial reactivity was detected using a high frequency ultrasound technique (4, 18). For this purpose, all patients were kept at rest in the supine position in a temperature-controlled room (22 C). The electrocardiogram was continuously monitored. Heart rate and finger arterial blood pressure

were measured by a noninvasive technique (Finapress, OMHEDA 2003, Englewood, CO) that uses the unloaded principle and has been shown to be as accurate as intraarterial blood pressure measurements (19). The left arm was immobilized in the extended position to allow consistent access to the brachial artery for imaging. Brachial artery diameter and flow velocity were imaged using a 7.5-MHz linear array transducer ultrasound system (Apogee CX 200, Interspec ATL, Ambler, PA). Brachial arterial diameter and blood flow velocity were recorded twice at an interval of 1 min. After that, a blood pressure cuff was placed over the ipsilateral upper arm just above the transducer, inflated for 5 min at 200 mm Hg, and then suddenly deflated. Blood flow velocity was measured immediately after cuff deflation (within 20 s), and brachial arterial diameter and flow were measured at 1-min intervals for 5 min. All images were recorded on videotape for subsequent off-line analysis on the same instrument by a single observer blinded to the conditions under which the ultrasonic images were obtained. Vit.E or placebo treatment lasted 24 h before the brachial reactivity was detected.

Analytical techniques

All blood samples were drawn into dark test tubes to which lithium heparin (10 U/mL) was added and were immediately centrifuged at 4 C. Plasma glucose and indexes of oxidative stress were immediately determined. All other blood samples were frozen at -20 C for further metabolite and hormone determinations.

Plasma glucose was determined by the glucose oxidase method (glucose autoanalyzer, Beckman Coulter, Inc., Fullerton, CA). Plasma insulin was determined by a commercial double antibody solid phase RIA (Linco Research, Inc., St. Charles, MO; coefficient of variation, 4.8 ± 0.3%; cross-reactivity with proinsulin, 0.2%). Plasma free fatty acid concentrations were determined by spectrophotometric methods (20). Plasma high density lipoprotein cholesterol concentrations were determined after precipitation of low density lipoprotein (LDL) and very low density lipoprotein with dextran sulfate and magnesium chloride (21). Commercial enzymatic methods were used in the determination of serum total cholesterol (Monotest, Roche Molecular Biochemicals, Milan, Italy; coefficient of variation, 3.8 ± 0.9%) (22) and triglycerides (Peridecrome, Roche Molecular Biochemicals; coefficient of variation, 4.1 ± 0.6%) (23). Serum LDL cholesterol levels were calculated using the Friedwald formula (24). Serum oxidative stress was measured as the reaction products of malondialdehyde (TBARS) with thiobarbituric acid (25). In this reaction, to prevent artificial autooxidation, *t*-butyl-4-hydroxyanisole (Sigma, St. Louis, MO) in a final concentration of 10 μmol/L was added to the specimens. The storage period of the specimens was no longer than 3 weeks before performing the assays. Each value was the result of the mean of three samples each one assayed in triplicate. LPO were measured according to the method of Yagi (26) and adjusted for plasma total cholesterol and triglycerides. Plasma GSH and GSSG concentrations were determined in the fasting state and at the end of each test according to the method of Beutler *et al.* (27). The plasma total antioxidant capacity was estimated by the 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation decolorization assay, using Trolox as a standard (TEAC) (16). In this assay, 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid is made to react with potassium persulfate in the absence or presence of standards and samples, and absorbance is read at 734 nm. A value of 1 TEAC in a sample is defined as a concentration that is equivalent to 1 mmol/L Trolox. The total fasting plasma Vit. E concentration was measured using the reverse phase high pressure liquid chromatography method (28).

Reproducibility of brachial reactivity indexes

Intraobserver variability for measuring brachial arterial diameter and flow was assessed by comparing a minimum of three separate baseline measurements in each patient. The coefficient of variation for baseline arterial diameter was 2.0%, and that for baseline arterial diameter was 9.7%. These values were not dissimilar from those reported previously (17, 18).

Calculation and statistical analyses

Statistical analyses were performed using SOLO (BMDP, Cork, Ireland) software package. Because the distribution of plasma insulin and tryglycerides concentrations and HOMA index are extremely skewed, each value was log-transformed to improve normality for statistical testing and back-transformed for presentation in tables and figures. All values are presented as the mean ± SD. The percent change was calculated with baseline values equal to 100%. ANOVA with Scheffe's test was used to calculate differences between the two study groups. Pearson product-moment correlations were made. Partial correlation allowed investigating the relationships between brachial reactivity and degree of oxidative stress (plasma GSH/GSSG ratio, TEAC, TBARS, and LPO concentrations) as well as plasma Vit. E levels independently of other covariates.

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All patients were adults, were slightly overweight with a prevalent central fat distribution, and had good metabolic control (Table 1). Both groups had similar baseline anthropometric, metabolic, and cardiovascular variables, which were unaffected by placebo and Vit.E treatments.

As far as oxidative stress indexes are concerned (Table 2), baseline GSH/GSSG, TBARS, LPO, and TEAC levels were not different between the two groups. Furthermore, all of these indexes remained unchanged at the end of placebo treatment. In contrast, Vit.E treatment significantly increased GSH/GSSG and TEAC and reduced TBARS and LPO levels (Table 2).

View this table: [in this window] [in a new window]	**Table 2.** Changes in oxidative stress indexes in the study groups

At baseline, Mgi and Ca^{2+} levels did not differ in the groups; at the end of the treatment period, Mgi was significantly higher in the Vit.E than the placebo group, whereas Ca^{2+} levels had opposite trend (Table 2).

Baseline hemodynamic data did not show any difference in brachial arterial diameter (3.52 ± 0.39 *vs.* 3.49 ± 0.43 mm; *P* = NS) and brachial arterial flow (81 ± 24 *vs.* 86 ± 22 mm/min; *P* = NS) between placebo and Vit.E groups. The percent change in brachial arterial flow and diameter was not different in the two study groups at baseline (Fig. 1). Vit.E treatment did not affect the percent change in brachial artery flow, whereas a significant increase in the percent change in brachial diameter was found. In contrast, placebo treatment was ineffective on brachial reactivity indexes (Fig. 1).

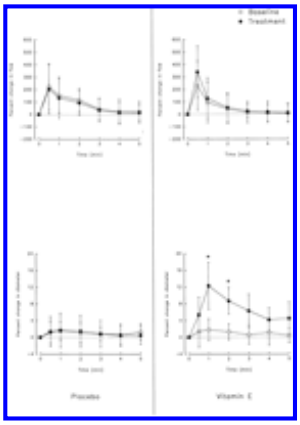

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Figure 1. Percent change in brachial artery flow and diameter at baseline (○) and at the end of the treatment period (•) in both placebo and Vit.E groups. *, $P < 0.03$.

In the Vit.E group, the percent change in brachial arterial diameter correlated positively with the percent increase in GSH/GSSG and TEAC and the percent decrease in TBARS and LPO (Fig. 2). Furthermore, significant positive correlations between the percent change in brachial arterial diameter and either the percent increase in Mgi or the percent decrease in Ca^{2+} were found (Fig. 3). The relationship between brachial reactivity and oxidative stress indexes was independent of age, sex, body mass index, and waist/hip ratio ($P < 0.03$ for all) as well as independent of hemoglobin A_{1C}, plasma LDL cholesterol, and HOMA index ($P < 0.04$ for all). Nevertheless, the relationship between percent change in brachial artery diameter and oxidative stress indexes (GSH/GSSG, TEAC, TBARS, and LPO) was no longer significant after adjustment for the percent change in Mgi and Ca^{2+}.

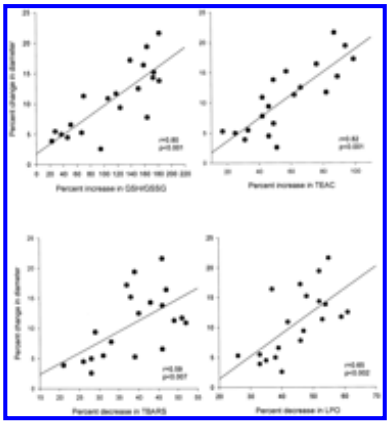

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Figure 2. Simple correlations between the percent change in diameter and the percent change in GSH/GSSG, TBARS, LPO, TEAC, and intracellular magnesium and calcium concentrations at the end of Vit.E treatment (n = 20).

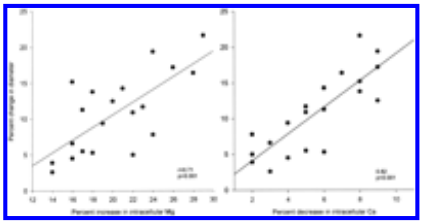

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Figure 3. Simple correlations between the percent change in diameter and the percent change in intracellular magnesium and calcium concentrations at the end of Vit/E treatment (n = 20).

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Our study demonstrates that chronic Vit.E administration improves brachial reactivity, reduces plasma oxidative stress indexes, and regulates intracellular cation content in type II diabetic patients. All of these phenomena were also correlated.

The evaluation of flow-mediated vasodilatation of the brachial artery has been previously suggested to be an index of endothelial dysfunction and early atherosclerosis (4). In fact, this technique, using high frequency ultrasound and hyperemia induced by a period of blood pressure cuff arterial occlusion, allows accurately determining the flow and diameter of a large artery in a noninvasive manner (18). In this way, impaired vascular reactivity has been found in many states involving endothelial dysfunction, such as hypercholesterolemia (29), hypertriglyceridemia (30), and diabetes mellitus (31).

It is important to point out that in diabetic patients, the presence of vascular disease is a problem accounting for the majority of the clinical complications leading to increased mortality (1). An exaggerated oxidative stress degree has been postulated as the link between diabetes mellitus and endothelial function (32). In fact, in these patients hyperglycemia (33), hyperinsulinemia (34, 35), and hypertriglyceridemia (36) lead to increased oxidative stress, which, in turn, might be responsible for an inactivation of NO. As it is now widely demonstrated that flow-dependent changes in arterial diameter are mediated by endothelium-dependent mechanisms, *i.e.* by the availability of NO (37, 38), the presence of elevated oxidative stress in diabetic patients results in endothelial dysfunction (3).

Once this hypothesis is accepted, antioxidant agents could be able to restore endothelial function. Actually, treatment with antioxidant has been found to improve endothelial function in animal models of hypercholesterolemia (4) and diabetes (5). In contrast, there are few studies in humans (6, 7, 39). Vit. C administration has been found to improve endothelium-dependent vasodilatation in patients with either type I (6) or type II (7) diabetes mellitus. Notwithstanding, these studies evaluated only the acute effect of intraarterial administration of Vit.C on endothelial function, measured by strain gauge plethysmography. From this point of view, our study was different from those previously reported, because endothelial dysfunction was assessed by brachial reactivity, and the effect of chronic Vit. E administration in type II diabetic patients was evaluated.

The favorable action of Vit.E on brachial reactivity shown in our study could be mainly due to two different mechanisms. Firstly, Vit.E is a potent scavenger of superoxide and other reactive species. In diabetes mellitus the presence of hyperglycemia leads to an abnormal production of superoxide anion and hydroxyl radicals and to a decreased VitE level in tissue and blood (8); in contrast, chronic Vit. E administration restores plasma antioxidant defenses such as the GSH/GSSG ratio (40). Thus, chronic Vit.

E administration may lower the quenching effect of free radicals on NO. Lastly, Vit.E may act through intracellular cation content. An altered intracellular steady state concentration of calcium and magnesium ions has been postulated to act as a final common pathway to regulate cellular glucose homeostasis, insulin sensitivity, peripheral vascular tone, and blood pressure (41). Increased oxygen free radical production is associated with low intracellular magnesium concentrations (9), and prior magnesium depletion makes cells more sensitive to oxidative damage (42). Vit.E has been demonstrated to protect against magnesium deficiency-induced myocardial injury (43) and magnesium deficiency-associated cerebral vascular damage (44). On the other hand, the effect of Vit.E on intracellular magnesium content leads to a reduction in intracellular calcium content, thus resulting in improved smooth vascular cell relaxation.

An unexpected finding of our study was that chronic Vit.E administration did not improve insulin resistance in a significant manner. Such a result might be in contrast with previous ones (45, 46) demonstrating a beneficial action of Vit.E. Nevertheless, differences in Vit.E dose (900 *vs.* 600 mg/day) and the technique used for assessing insulin action (clamp *vs.* HOMA) could account for this discrepancy.

In conclusion, our study demonstrates that chronic administration of Vit.E improves brachial artery reactivity in patients with type II diabetes mellitus. Such an effect seems to be mediated by a reduction in oxidative stress and a regulation of intracellular calcium and magnesium contents. Further studies will clarify the molecular mechanism(s) of this effect.

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