A study on the action of vitamin E supplementation on plasminogen activator inhibitor type 1 and platelet nitric oxide production in type 2 diabetic patients

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Abstract Background and aim: Type 2 diabetic (T2DM) patients show decreased fibrinolysis, mainly linked to high plasminogen activator inhibitor type 1 (PAI-1) production, together with a reduced bioavailability of nitric oxide and an impairment in Na+/K+-ATPase activity possibly involved in increased cardiovascular risk. Vitamin E is the major natural lipid-soluble antioxidant in human plasma. The present work was conducted in order to measure PAI-1, ICAM and VCAM-1 plasma levels, platelet nitric oxide production and membrane Na+/K+-ATPase activity in type 2 diabetic subjects treated with vitamin E (500 IU/day) for 10 weeks and then followed for other 20 weeks.

Methods and results: Thirty-seven T2DM patients (24 males and 13 females) were studied. None of them were affected by any other disease or diabetic complications. Significant differences were detected for PAI-1 antigen (p < 0.001), PAI-1 activity (p < 0.001), nitric oxide (NO) production (p < 0.001), and Na+/K+-ATPase activity (p < 0.001) among the 4 phases of the study. A significant decrease both in ICAM and VCAM-1 plasma levels was also found at the 10th week compared with baseline (respectively p < 0.001 and p < 0.05).

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Conclusion: Our data suggest that vitamin E counteracts endothelial activation in T2DM patients possibly representing a new tool for endothelial protection. © 2007 Elsevier B.V. All rights reserved.

Introduction

Plasminogen activator inhibitor type 1 (PAI-1) is considered the most important physiologic fibrinolysis inhibitor. It plays also an essential role in the regulation of fibrinolysis, rapidly reacting and inactivating the tissue plasminogen activator (tPA) [1,2]. It is a serine protease, mainly produced by the liver and by endothelial cells [3], that binds to the active site of both tPA and urokinase plasminogen activator (uPA) thereby neutralizing their activity. Its increase in type 2 diabetes mellitus determines a fibrinolytic impairment and it is linked with premature atherosclerosis and increased morbidity and mortality, caused by myocardial infarction, cerebrovascular disease and peripheral vascular disease [4,5].

Different factors and pathways have been proposed to affect PAI-1 plasma levels in diabetic patients, such as hyperinsulinemia [6,7] insulin resistance [8], hypertriglyceridemia [9], glucose concentration [10] free radical generation [11] and nitric oxide (NO) production [12].

NO is a paracrine mediator exerting a number of physiologic actions in various organs, including vascular endothelium where it acts as a potent vasodilator [13]. NO is synthesized as a by-product of conversion of its physiologic precursor l-arginine (l-arg) to l-citrulline. This reaction is catalyzed by a family of enzymes known as NO synthases (NOS). Three NOS isoforms (neuronal, nNOS, NOS1; inducible, iNOS, NOS2; endothelial, eNOS, NOS3) have been identified in mammalian tissues. Platelets contain the same NOS isoform as endothelial cells, so that it might be assumed that they are a suitable model for the study of endothelial NOS [14].

The role of NO on PAI-1 production is still under debate. Some authors found that nitric oxide suppresses vascular PAI-1 expression by inhibition of PAI-1 synthesis in vascular smooth muscle cells [15] and by reduction of PAI-1 release from platelets [16]. Instead, other researchers found a dual effect of NO on PAI-1 expression in endothelial cells [17]. In particular, NO endogenously generated by NOS can potentiate PAI-1 expression, while NO exogenously released from NO donors inhibits its generation. No in vivo data are available to confirm or disclaim these findings, leaving the problem of whether NO up- or down-regulates PAI-1 synthesis still open. Type 2 diabetes mellitus (T2DM) actually presents as both impaired NO production [18] and increased PAI-1 plasma levels [19], as well as an elevation in the production of cellular adhesion molecules such as the vascular cell adhesion molecule-1 (VCAM-1) and the intracellular adhesion molecule (ICAM) [20].

Vitamin E is the major natural lipid-soluble antioxidant in human plasma, where it is associated primarily with LDL [21]. As Vitamin E supplementation has been demonstrated as able to decrease PAI-1 levels [22,23] and to increase endothelium-dependent vasorelaxation [24], the present work was conducted in order to verify whether in vivo vitamin E treatment may affect platelet NO production together with PAI-1 plasma levels in type 2 diabetes mellitus. Moreover, VCAM-1 and ICAM were also measured in the same group of patients as a recent study by Desideri and collaborators reported that vitamin E supplementation reduces plasma VCAM-1 and increases NO concentration in hypercholesterolemic patients [25]. Finally, platelet Na+/K+-ATPase activity was determined in the same group of subjects, as in a previous study from our group we found a significant relationship between platelet Na+/K+-ATPase and NOS activity in both type 1 and type 2 diabetic patients [18].

Methods

Patients and blood samples

Thirty-seven T2DM patients (24 males and 13 females; mean age ± SD: 62.4 ± 7.9) were studied after informed consent was obtained. The study was performed in accordance with the principles of the Declaration of Helsinki as revised in 1996. None of the patients were affected by any other disease. Patients were selected according to the following criteria: a) dietetic therapy only; b) stable and satisfactory glycemic control. For this purpose fasting and post-prandial glycemia, HbA1c levels as well as glycosuria were analyzed during the 3 months preceding the trial. Only those patients showing stable glycemic values (fasting glycemia and glycosuria variation <15%; post-prandial glycemia variation <25% and HbA1c <7.5%) were included in the study. Besides, the selected patients were non-smokers, consumed a Mediterranean diet and none of them had an alcohol abuse history or took
any drugs known to lower lipids and interfere with the coagulation and antioxidant systems. A slightly hypocaloric diet consisting of 1800 Kcal/day; 18% proteins, 28% lipids, 54% carbohydrates and 34 g of fiber was prescribed 6 months before the beginning of the study. Interviews were performed at three month intervals to verify that patients were correctly following the diet prescribed and that they did not change the antioxidant intake. Overnight fasting venous blood samples were collected without hemostasis, in a resting state, between 08:00 and 09:00 h to overcome the diurnal variation of PAI-1, and immediately processed. Plain, EDTA, citrate and strong acidic citrate added tubes were used.

After 10 min of centrifugation (2500 × g) at 4°C, the middle layer of the plasma was rapidly pipetted off and stored at −80°C.

Study design

This study consisted of a 10 week pre-treatment period, a 10 week treatment period and a 20 week post-treatment period.

Pre-treatment study

The biological variation (CVb) of PAI-1 was evaluated before the treatment with vitamin E [22]. Specifically, $CV_b = (CV^2_t - CV^2_a)^{1/2}$, where $CV_b$ is the coefficient of biological variation, $CV_t$ is the total intra-individual PAI-1 variability and $CV_a$ is the coefficient of analytical variation which, based on the inter-assay CV of the quality control samples, was calculated to be 5.3% for PAI-1 antigen and 6.8% for PAI-1 activity determination.

Treatment

Before starting vitamin E treatment, platelet NO production, plasma membrane Na+/K+-ATPase activity, plasma ICAM and VCAM-1 and the main glyco-metabolic, lipidic and hemostatic parameters were assayed. Diabetic patients received 500 IU/day vitamin E (α-tocopherol acetate, E-VITUM, Molteni, Florence, Italy) for 10 weeks. During the 5th and 10th week, and after the 30th week of vitamin E intake, the same parameters were determined. The choice to protract the washout evaluation at the 30th week is due to the known low mobilization of vitamin E from peripheral tissues such as adipose tissue [26].

Determination of blood parameters

Blood concentrations of the main parameters of lipo- and glyco-metabolic balance (total cholesterol, HDL cholesterol, triglycerides and fasting glucose) were measured by standard procedures. Glycosylated hemoglobin was assayed by cation exchange chromatography and spectrophotometric detection (Diamat Analyzer, Bio-Rad). Serum concentration of fructosamines was determined spectrophotometrically (Unimate 3, Roche, Basel, Switzerland).

An immunoenzymatic method for PAI-1 antigen determination was used (Tintelize PAI-1, Biopool, Sweden). PAI-1 antigen measurement is able to detect active and latent forms of PAI-1 as well as tissue plasminogen activator (tPA)/PAI-1 and urokinase plasminogen activator (uPA)/PAI-1 complexes [22]. The PAI-1 activity was assayed by a chromogenic method (Spectrolyse™/fibrin, Biopool, Sweden), which determines non-complexed PAI-1 activity [22]. tPA antigen was assayed with an ELISA method (Tintelize tPA, Biopool, Sweden), while tPA activity determination was performed with a chromogenic method (Spectrolyse/fibrin, Biopool, Sweden). To minimize the analytical variation PAI-1 antigen and PAI-1 activity were analyzed in duplicate in the same plate at the end of the study.

Vitamin E was determined by an isocratic HPLC method employing a reversed-phase C-18 column and an ultraviolet (UV) detector after deproteination and extraction of samples [22]. The soluble VCAM-1 and ICAM-1 concentrations were quantified with the use of a commercially available sandwich enzyme immunosorbent assay kit (R&D Systems; Minneapolis, MN, USA). The samples were processed according to the instructions of the manufacturer. In brief, standards, samples, controls and conjugate were added to a 96-well microplate precoated with a monoclonal anti-VCAM-1 or ICAM-1 antibody. After 1.5 h of incubation and washing to remove any unbound substances, a substrate solution was added to induce a colored reaction product and the intensity of the color was measured by using a microplate reader (Wallac, Victor2) set to 450 nm with a correction wavelength of 630 nm.

Platelet NO production

NO released by the cells was measured in the intact platelets suspension as nitrite/nitrate, by the Griess reaction [27]. Briefly, in this assay equal amounts of 1% sulfanilic acid and 0.1% N-(1-naphthyl) ethylene diamante were added to the samples and the resulting absorbance was measured at 543 nm. Blank (background) was determined in each experiment utilizing medium incubated without cell. The amount of NO in each sample was determined using a standard
curve generated with known concentrations of NO; protein concentration was determined with the Bradford BioRad protein assay using serum albumin as a standard [28]. NO concentration was expressed as nmol NO/mg protein.

**Na⁺/K⁺-ATPase assay**

Na⁺/K⁺-activated Mg²⁺-dependent ATPase activity was determined in platelet plasma membranes obtained using the method of Enouf [29]. ATPase activity was assayed by incubating membranes at 37 °C in reaction buffer containing 5 mM MgCl₂, 140 mM NaCl, 14 mM KCl in 40 mM Tris–HCl, pH 7.7 as previously described [18]. The ATPase reaction was started by adding 3 mmol/L Na₂ATP. The inorganic phosphate (P₁) hydrolyzed from this reaction was measured according to Fiske and Subbarow [30]. ATPase activity assayed in the presence of 10 mM ouabain was subtracted from the total Mg²⁺-dependent ATPase activity to calculate Na⁺/K⁺-ATPase activity and was expressed as μmol P₁/mg protein per hour. Protein concentration was determined with the Bradford BioRad protein assay using serum albumin as a standard [28].

### Statistical analysis

All results are shown as means ± SD. Data were analyzed with the SPSS-Win program (version 11.5, SPSS Inc. Chicago, IL, USA). Repeated analysis of variance was used to analyze the differences among the phases of the study for the considered variables using Hotelling’s T tests to verify the profiles of the different assays. Multiple comparisons were performed, when appropriate. Pearson’s simple correlation was used to verify the association between PAI-1 and NO at baseline. Probability value less than 0.05 was considered statistically significant.

### Results

The main characteristics of the diabetic patients studied are listed in Table 1, which also shows the evaluated parameters before, during and after vitamin E supplementation. The increase in vitamin E at the 10th week is significant, which confirmed that vitamin E was correctly assumed and well assimilated.

Regarding the glycometabolic control, no significant effects were highlighted. In fact, fasting glycemia, glycylated hemoglobin and fructosamines did not show any variation during the trial. Regarding the lipidic parameters, no changes were found for total cholesterol, triglycerides, HDL cholesterol, as well as the hemocoagulative parameters tPA antigen and activity. A significant decrease both in ICAM and VCAM-1 plasma levels was found at the 10th week compared with baseline (Table 1, respectively p < 0.001 and p < 0.05).

Figs. 1, 2, 3 and 4 show, respectively, PAI-1 antigen and activity, platelet NO production and Na⁺/K⁺-ATPase activity evaluated during the trial. Significant differences were detected for PAI-1 antigen (Fig. 1; p < 0.001), PAI-1 activity (Fig. 2; p < 0.001), NO production (Fig. 3; p < 0.001) and Na⁺/K⁺-ATPase activity (Fig. 4; p < 0.001) among the four phases of the study. In particular, a significant decrease was found for PAI-1 antigen between baseline and the 5th and 10th week (p < 0.05) followed by an increase at the 30th period.

### Table 1  Blood and anthropometric parameters at the different period of evaluation

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>5th week</th>
<th>10th week</th>
<th>30th week</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62.4 ± 7.9</td>
<td>62.4 ± 7.9</td>
<td>62.4 ± 7.9</td>
<td>62.4 ± 7.9</td>
<td>0.159</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>6.5 ± 6.5</td>
<td>6.5 ± 6.5</td>
<td>6.5 ± 6.5</td>
<td>6.5 ± 6.5</td>
<td>0.961</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>27.5 ± 3.4</td>
<td>27.5 ± 3.2</td>
<td>27.6 ± 3.3</td>
<td>27.5 ± 3.3</td>
<td>0.159</td>
</tr>
<tr>
<td>Vitamin E (μg/ml)</td>
<td>6.1 ± 0.4</td>
<td>8.5 ± 0.5</td>
<td>9.7 ± 0.6</td>
<td>5.6 ± 0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>7.05 ± 1.71</td>
<td>6.72 ± 1.35</td>
<td>7.28 ± 1.55</td>
<td>7.61 ± 1.52</td>
<td>0.354</td>
</tr>
<tr>
<td>Fructosamines (μmol/L)</td>
<td>353 ± 51</td>
<td>370 ± 55</td>
<td>361 ± 51</td>
<td>345 ± 32</td>
<td>0.173</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.5 ± 0.8</td>
<td>6.6 ± 0.7</td>
<td>6.5 ± 0.7</td>
<td>6.6 ± 0.8</td>
<td>0.168</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.96 ± 1.14</td>
<td>6.01 ± 1.09</td>
<td>6.21 ± 1.41</td>
<td>6.11 ± 1.16</td>
<td>0.526</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.26 ± 0.33</td>
<td>1.21 ± 0.29</td>
<td>1.23 ± 0.28</td>
<td>1.26 ± 0.26</td>
<td>0.479</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.63 ± 0.96</td>
<td>1.75 ± 1.11</td>
<td>1.77 ± 0.84</td>
<td>1.69 ± 0.84</td>
<td>0.894</td>
</tr>
<tr>
<td>tPA antigen (ng/ml)</td>
<td>14.6 ± 4.8</td>
<td>13.1 ± 3.9</td>
<td>13.0 ± 5.4</td>
<td>14.3 ± 4.1</td>
<td>0.147</td>
</tr>
<tr>
<td>tPA activity (IU/ml)</td>
<td>3.7 ± 1.4</td>
<td>3.3 ± 1.0</td>
<td>2.8 ± 1.2</td>
<td>3.5 ± 1.3</td>
<td>0.079</td>
</tr>
<tr>
<td>ICAM (μg/l)</td>
<td>368.5 ± 91.9</td>
<td>297.8 ± 49.2</td>
<td>517.0 ± 161.9</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>VCAM-1 (μg/l)</td>
<td>698.7 ± 483.0</td>
<td>517.0 ± 161.9</td>
<td>517.0 ± 161.9</td>
<td>&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

Means ± standard deviations are shown.
A similar behavior was found for PAI-1 activity (Fig. 2; \( p < 0.05 \)). On the contrary, NO showed a significant increase at the 5th, 10th and 30th week vs. baseline \( (p < 0.05) \) although a reduction was observed at the 30th week vs. the 10th week (Fig. 3; \( p < 0.05 \)). The same trend was observed for \( \text{Na}^+/\text{K}^+ \)-ATPase activity: a significant increase at the 5th, 10th and 30th week vs. baseline \( (p < 0.05) \) but a reduction was found at the 30th week vs. the 10th week (Fig. 4; \( p < 0.05 \)).

**Discussion**

Type 2 diabetic subjects have an increased risk for cardiovascular disease. This risk is, at least in part, attributable to a reduced fibrinolytic activity mainly due to PAI-1 overproduction and to endothelial dysfunction caused by the decreased bioavailability of NO. As new therapeutic strategies are aimed to reduce this risk by increasing bioavailability of NO, it is important to study the relations existing in vivo between these two molecules.

Our data confirm that vitamin E supplementation causes a decrease of PAI-1 levels and demonstrate that this reduction is accompanied by an increase of platelet NO production and platelet membrane \( \text{Na}^+/\text{K}^+ \)-ATPase activity in type 2 diabetic subjects.

Figs. 1–4 clearly show the contemporaneous significant variations of these parameters at the 5th and the 10th week followed by a restore towards the baseline values. The mechanism by which vitamin E decreases PAI-1 levels has not been completely clarified. It is not likely that vitamin E acts on PAI-1 overproduction in T2DM through the known causes mentioned in the Introduction. In fact, glyco and lipo-metabolic parameters did not show any variation during or after the trial (Table 1). Moreover, it has been recently demonstrated that vitamin E-induced improvements of the antioxidant balance and/or anti-inflammatory activity are not related to PAI-1 production [32].
Direct effects of vitamin E on PAI-1 might be hypothesized, as vitamin E is able to inhibit protein kinase C and, subsequently, MAP kinase activation that mediates the generation of PAI-1 in endothelial cells [33]. Another possibility is that vitamin E can inhibit the NF-κB protein transcription, thus decreasing PAI-1 production [34].

Murakami et al. [35] demonstrated that a weight reduction by caloric restriction improves fibrinolytic parameters, probably through reduction of adipose tissue. Our patients did not modify their BMI during or after the trial (Table 1) so the prescribed diet was not the cause of modification of the parameters investigated. A Mediterranean-inspired diet has been demonstrated to affect the inflammatory process and endothelial indexes such as vasoregulation and vascular endothelial growth factors in healthy subjects. [36]. Minor components of olive oil strongly reduce in vitro cell surface expression of ICAM-1 and VCAM-1 adhesion molecules involved in early atherogenesis [37]. Part of the beneficial effects of the Mediterranean diet in atherosclerosis might be related to its effects on inflammation and endothelial function [38]. However, the Mediterranean diet was started by our patients 6 months before the beginning of the study, so that modifications possibly due to dietary changes can be ruled out, as demonstrated by the lack of modifications in BMI, glycemic control and plasma lipid parameters during the study.

Regarding the relation between NO and PAI-1, it might be hypothesized on the basis of our results that NO may contribute to regulating the production of PAI-1, probably through a mechanism of down-regulation. Our data are consistent with a previous study which reported that administration of glutathione in patients with T2DM increases the platelet constitutive NO synthase activity and reduces PAI-1 levels [39] and with previous reports on the suppression of vascular PAI-1 expression by NO through inhibition of PAI-1 synthesis in vascular smooth muscle cells and by reduction of PAI-1 release from platelets [40]. In fact, our T2DM patients showed an increased platelet NO production contemporaneously with decreased PAI-1 production during vitamin E supplementation. Nevertheless, we cannot exclude that the decreased PAI-1 production is caused by a direct vitamin E-dependent modulation of PAI-1 gene. In fact, a report by our group [23] has shown that vitamin E supplementation in type 2 diabetic patients modifies PAI-1 levels in a different way according to the 4G/5G polymorphism of PAI-1, which has been previously reported to be linked to a higher risk of cardiovascular complications.

Vitamin E is not directly able to scavenge superoxide radicals because of the low reaction rate constant ($k_2 = 4.96 \times 10^3 M^{-1} s^{-1}$). Thus, vitamin E may decrease intracellular oxidative stress by secondary mechanisms. We can then infer that the decrease in oxidative stress resulting from antioxidant vitamin therapy may improve the bioavailability of endothelium derived NO (EDNO). Indeed, increases in the production of superoxide anion, typical of diabetes, decreases bioavailable NO to form peroxynitrite ($ONOO^-$) which, in turn, enhances the production of superoxide anion by oxidizing the endothelial NOS cofactor tetrahydrobiopterin.

In the present study vitamin E oral administration caused an increase in $Na^+/K^+$-ATPase activity. This finding is consistent with previous studies reporting that Vitamin E protects the membrane-bound enzyme activities from damages caused by lipid peroxidation, which is present in diabetes mellitus [41].

Another interesting finding of our report is the reduction of plasma ICAM and VCAM-1 levels observed after vitamin E supplementation. Vitamin E, as well as other antioxidants, might be able to inhibit the antioxidant-sensitive control mechanisms involved in VCAM-1 and ICAM expression in human endothelial cells [42]. Indeed, increased circulating ICAM and VCAM-1 concentration suggests that an endothelial activation is present in these patients and that it reversed after vitamin E oral treatment. In our report we did not investigate the mechanisms leading to decreased ICAM and VCAM-1 levels in diabetic patients after vitamin E supplementation. However, we could speculate that increased NO levels might contribute to decreased ICAM and VCAM-1 levels in diabetic subjects. In fact, it has been previously reported that NO regulates the activation of NF-B through its interaction with reactive oxygen species (ROS). NF-B in turn is able to regulate the ICAM and VCAM-1 genes in vascular endothelium [43].

In contrast to the increased platelet NO production observed during vitamin E supplementation in the present study, Beckman et al., [44] failed to find improvements in endothelium-dependent vasodilation in T2DM after oral antioxidant vitamins treatment (both vitamin C and E). In our opinion, this fact might be explained by different selection criteria used for the patients. Thus, a number of influential factors such as age, BMI, pharmacologic treatment, different diet habits attributable to ethnicity might affect the studied population and lead to a lack of improvement in vascular functions in such patients.
Vitamin E supplementation modify PAI-1 and NO production in T2DM

On the contrary, our results are consistent with a previous study by Paolisso et al. [24], which demonstrated that vitamin E administration improves brachial reactivity, reduces plasma oxidative stress indexes, and regulates intracellular cation content in T2DM.

However, it must be underlined that a potential limitation of the present study is the lack of a control arm treated with a placebo and of a double-blind randomization. Because of these limitations, our results can be considered highly suggestive of an effect of vitamin E supplementation on endothelial function in T2DM, but also indicates the need for further placebo-controlled studies on this issue.

In conclusion, our data suggest that vitamin E counteracts endothelial activation in T2DM patients, as vitamin E supplementation reduces PAI-1 activity, ICAM and VCAM-1 concentrations and increases NO production and plasma membrane Na⁺/K⁺-ATPase activity. Vitamin E might therefore be effective in preventing early endothelial damage in type 2 diabetes mellitus, possibly representing a new tool for endothelial protection.

References


