α-Tocopherol Supplementation Decreases Plasminogen Activator Inhibitor-1 and P-Selectin Levels in Type 2 Diabetic Patients

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OBJECTIVE — Type 2 diabetic subjects have an increased propensity to premature atherothrombosis. α-Tocopherol (AT), a potent antioxidant, has anti-inflammatory properties at high doses. The aim of the study was to test the effect of natural (RRR)-AT supplementation (1,200 IU/day) on markers of thrombosis, plasminogen activator inhibitor-1 (PAI-1), and soluble P-selectin (sP-selectin) in type 2 diabetic patients with and without macrovascular complications (MVCs) compared with matched control subjects.

RESEARCH DESIGN AND METHODS — The volunteers comprised type 2 diabetic patients with (n = 23) and without (n = 24) MVCs and matched control subjects (n = 25). Plasma levels of PAI-1 and P-selectin were assayed at baseline, after 3 months of supplementation, and after a 2-month washout phase.

RESULTS — Both diabetic groups had significantly increased levels of PAI-1 compared with control subjects (P < 0.025), whereas only type 2 diabetic patients with MVCs had significantly elevated levels of sP-selectin compared with control subjects. AT supplementation significantly lowered levels of PAI-1 and sP-selectin in all three groups. The reduction in PAI-1 levels with AT supplementation was significantly greater in type 2 diabetic patients with MVCs than in those without MVCs (P = 0.005).

CONCLUSIONS — Thus, AT therapy decreases markers of thrombosis in diabetic patients and control subjects and could be an adjunctive therapy in the prevention of atherosclerosis.

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Type 2 diabetes is associated with accelerated atherosclerosis and atherothrombosis. Macrovascular complications (MVCs) are the major cause of morbidity and mortality in type 2 diabetes (1,2). Several factors contribute to this proatherogenic and prothrombotic state, including abnormalities in fibrinolysis and increased platelet activity (3–5). Plasminogen activator inhibitor-1 (PAI-1) is a key regulator of fibrinolysis and inhibits breakdown of fibrin by inhibiting tissue plasminogen activator (tPA). Decreased fibrinolysis, primarily caused by increased PAI-1 activity, has been demonstrated in patients with coronary artery disease (6). Elevated PAI-1 is considered a strong risk factor for cardiovascular disease (CAD) and has been shown in some reports to be elevated in type 2 diabetes (7–10). Also, PAI-1 has been shown to be increased in coronary plaques in patients with type 2 diabetes (11). Furthermore, PAI-1 levels correlate with many variables that cosegregate with the metabolic syndrome, such as BMI, waist-to-hip ratio, insulin, triglycerides, and apolipoprotein B levels (12,13). There are scant data on modulation of PAI-1 levels in type 2 diabetes, especially with antioxidant therapy.

An early event in atherogenesis is attachment of leukocytes to the endothelium (14). Soluble cell adhesion molecules that are shed from activated cells are thought to reflect the functional status of phagocytes, platelets, and endothelial cells. Increasing evidence supports the role of plasma levels of the soluble cell adhesion molecules (CAMs) soluble intracellular adhesion molecule-1 (sICAM-1), soluble vascular CAM-1 (sVCAM-1), E-selectin, and P-selectin as emerging molecular markers of atherosclerosis (15–17). P-selectin mediates adhesion of platelets to endothelial cells during inflammation and thrombosis and is largely contained in the platelet α-granules, and to some extent in the Weibel-Palade bodies of endothelial cells, and is released on activation (18,19). AT therapy (600 IU/day for 2 weeks) has been shown to significantly reduce levels of soluble P-selectin in hypercholesterolemic patients (20). However, there is no data on the effect of AT supplementation on P-selectin levels in patients with type 2 diabetes.

α-Tocopherol (AT) is a potent lipid-soluble antioxidant. AT supplementation, in addition to decreasing LDL oxidative susceptibility and F2-isoprostanes (a direct measure of in vivo lipid peroxidation), has also been shown to be antiatherogenic, as evidenced by decreased...
platelet aggregation, improved endothelial function, and decreased monocyte proatherogenic activity (21,22). In a recent study, we showed that monocytes from type 2 diabetic subjects with and without MVCs had increased proatherogenic activity, as evidenced by increased levels of superoxide anion, increased levels of the proinflammatory cytokines interleukin (IL)-1β and -6, and increased adhesion to human endothelium compared with age-, sex-, and BMI-matched control subjects (23,24). Also, supplementation with RRR-AT (1,200 IU/day for 3 months) significantly attenuated monocyte proatherogenic activity (superoxide, IL-1β, tumor necrosis factor-α, and adhesion to endothelium) and the soluble CAMs, sICAM, sVCAM, and E-selectin as well as high-sensitivity C-reactive protein (CRP; the prototypic marker of inflammation) compared with baseline and a 2-month washout phase (23,24). This high dose of AT was chosen because we have previously shown in healthy nondiabetic subjects that this dose was beneficial with regards to monocyte proatherogenic activity (25). Owing to the critical role of thrombosis in diabetes, we report on levels of PAI-1 and P-selectin at baseline and after AT supplementation in type 2 diabetic patients and matched control subjects.

RESEARCH DESIGN AND METHODS — A total of 75 subjects were recruited and divided into three groups: type 2 diabetic subjects without and with MVCs and age-, sex-, and BMI-matched healthy control subjects. All subjects volunteering to participate who fulfilled the selection criteria gave informed consent, and the study protocol was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center. Exclusion criteria were smoking, ingestion of antioxidant supplements and vitamins, lipid-lowering drugs, β-blockers, nonsteroidal anti-inflammatory drugs, renal or liver dysfunction, or alcohol consumption >1 oz/day. Details of inclusion criteria and the salient characteristics of the subjects has been previously reported (23,24). Antidiabetic therapies included metformin, glyburide, and insulin. Criteria for macrovascular disease in diabetic patients included evidence of cardiovascular disease (CVD) (clinical presentation and electrocardiographic evidence of myocardial infarction, or positive stress tests or coronary angiography) or cerebrovascular disease (stroke or transient ischemic attacks, or magnetic resonance imaging evidence) or peripheral vascular disease (amputation, intermittent claudication, evidence of vascular disease with color-flow Doppler by B-mode ultrasound, amputation, or ankle-brachial index <0.8 and toe pressures <45 mmHg). Of the type 2 diabetic patients with MVC, 12 had evidence of CVD, 4 had evidence of cerebrovascular disease, 4 had peripheral vascular disease (PVD), 2 had both CVD and PVD, and 1 had a cerebrovascular accident and PVD. Before entry, subjects had a complete laboratory evaluation, including complete blood count, routine chemistry, and spot urine for microalbumin and creatinine. We collected 24-h urine samples if a spot microalbumin/creatinine ratio was >30 mg/g creatinine. A total of 72 subjects completed the study.

Fasting blood was drawn at baseline, after a 3-month supplementation with RRR-AT (1,200 IU/day) and after a 2-month washout phase. AT levels in plasma and monocytes were measured after ethanol precipitation and hexane extraction by reverse-phase high-performance liquid chromatography as described previously (24,25). HbA1c levels were measured in all subjects at all three time points by automated affinity chromatography as reported previously (24,25). PAI-1 levels in plasma were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using reagents from American Diagnostica. Soluble levels of P-selectin in plasma were measured by a quantitative sandwich immunoassay using reagents from R&D Systems. The intra-assay coefficients of variation for the PAI-1 and P-selectin assays were 9 and 4.5%, respectively. LDL was isolated, and copper-catalyzed LDL oxidation was performed by measurement of conjugated dienes (24). The lag phase of conjugated dienes was computed as described previously (24). Urinary F2-isoprostanes were measured by ELISA as described previously (26). CRP levels were measured using a high-sensitivity assay as described previously (23), and monocyte IL-1 levels were measured by sandwich ELISA as described previously (24). Kruskal-Wallis ANOVA was performed to assess differences in responses after supplementation in the different parameters between the three groups. Wilcoxon’s signed-rank tests were performed for within-group comparisons and Mann Whitney U tests for between-group comparisons; the level of significance was set at 0.025 to adjust for multiple testing. Spearman’s rank correlation was performed to examine associations between the parameters tested. All statistical analyses were performed by the biostatistician at the General Clinical Research Center using SAS software (SAS Institute, Cary, NC).

RESULTS — All subjects were matched for age, BMI, and lipids, as reported previously (24). Levels of HbA1c were significantly increased in both diabetic groups compared with control (4.5 ± 0.4 for control subjects; 8.1 ± 2.2 and 8.4 ± 2.3 for type 2 diabetic patients without and with MVC, respectively, P < 0.001 compared with control subjects). However, AT supplementation did not have any significant effect on HbA1c levels in any of the three groups. The lipid and lipoprotein profiles of subjects are depicted in Table 1. There were no significant differences in the lipid and lipoprotein levels among the three groups. Also, plasma lipids and lipoproteins did not change significantly in any of the three groups during the period of the study.

In this study, we show that compared with matched control subjects, only the type 2 diabetic patients with MVC have significantly elevated levels of P-selectin (Table 2); although in type 2 diabetic patients, P-selectin levels were higher compared with control subjects, it escaped statistical significance (P = 0.027), using our higher threshold of significance, which adjusts for multiple testing (P < 0.025). However, when the diabetic groups were combined, the increase in soluble P-selectin levels was significant compared with control subjects (P < 0.01). Both diabetic groups had significantly elevated levels of PAI-1 compared with control subjects (Table 2). There were no significant differences in P-selectin and PAI-1 levels between the two diabetic groups.

After supplementation, plasma lipid-standardized AT rose 2- to 2.5-fold compared with baseline and returned to baseline after the washout phase in all three groups (23,24). AT supplementation at this dose was not reported to be associated with any side effects. AT sup-
α-Tocopherol and type 2 diabetes

Table 1—Lipid and lipoprotein profile of control subjects and type 2 diabetic patients

<table>
<thead>
<tr>
<th>Group</th>
<th>Control subjects</th>
<th>Type 2 diabetic patients</th>
<th>Type 2 diabetic patients with MVCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 months</td>
<td>3 months</td>
<td>5 months</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>197 ± 32</td>
<td>203 ± 32</td>
<td>194 ± 32</td>
</tr>
<tr>
<td>Total triglyceride (mg/dl)</td>
<td>116 ± 46</td>
<td>112 ± 48</td>
<td>110 ± 55</td>
</tr>
<tr>
<td>VLDL cholesterol (mg/dl)</td>
<td>17 ± 10</td>
<td>16 ± 11</td>
<td>16 ± 11</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>50 ± 15</td>
<td>47 ± 13</td>
<td>49 ± 13</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>130 ± 29</td>
<td>140 ± 29</td>
<td>129 ± 26*</td>
</tr>
</tbody>
</table>

Data are means ± SD. *P < 0.02 compared with 3 months.

plication significantly decreased levels of P-selectin and PAI-1 in all three groups compared with both baseline and washout phases (Figs. 1 and 2). The median reduction in levels of P-selectin was similar in all three groups (17, 28, and 25% for control subjects and type 2 diabetic patients without and with MVC, respectively). Whereas AT supplementation resulted in significant reductions in PAI-1 levels in all three groups, a significantly greater reduction in PAI-1 levels was observed in type 2 diabetic patients with MVC compared with those without MVC (16 vs. 5%, P = 0.005). There were no significant differences in PAI-1 and P-selectin levels between baseline and after the washout phase.

There was a significant correlation between levels of P-selectin as well as PAI-1 with levels of HbA1c when all the groups were combined. (P-selectin, r = 0.43, P < 0.005; PAI-1, r = 0.32, P < 0.01). Also, there was no significant correlation between reduction in PAI-1 and sP-selectin and reduction in markers of oxidative stress and inflammation after AT supplementation. Correlations between PAI-1 and plasma CRP, lag phase of conjugated dienes, F2-isoprostanes, and monocyte IL-1 were 0.17, 0.16, 0.15, and 0.11, respectively, P > 0.1. Also, correlations between reductions in P-selectin and plasma CRP, lag phase of conjugated dienes, F2-isoprostanes, and monocyte IL-1 were −0.17, −0.09, −0.26, and 0.16, respectively, P > 0.1.

CONCLUSIONS — There is little data on modulation of PAI-1 levels in type 2 diabetes. Recently, troglitazone has been demonstrated to significantly reduce levels of PAI-1 in diabetic subjects (27). In this report, for the first time, we report that therapy with an antioxidant AT, which has potent anti-inflammatory effects (23,28), significantly decreases levels of PAI-1 in both control and type 2 diabetic subjects with and without MVCs. Because troglitazone comprises AT as part of its molecular structure and has been shown to have antioxidant activity, it is conceivable that the effect obtained in the troglitazone study on PAI-1 could be attributed to AT; however, the effect of AT supplementation alone was not studied in that report (28). Two small reports have examined the effect of AT supplementation on PAI-1 levels in type 2 diabetes (29,30), and the results are conflicting. Skrha et al. (29) reported the effects of administration of AT (600 mg/day for 3 months) in 11 obese subjects with mild type 2 diabetes. Although levels of tissue plasminogen activator decreased, PAI-1 concentrations were not significantly changed after treatment with AT. Thus, AT was reported to aggravate hypofibrinolysis, as evidenced by an increase in the PAI-1-to-tPA ratio. However, in a detailed study, Bonfigli et al. (30) examined the effect of vitamin E supplementation (d-AT acetate, 500 IU/day for 10 weeks) on PAI-1 levels in 13 elderly type 2 diabetic patients under good glycemic control (aged between 60 and 70 years, HbA1c <7.5%, on diet therapy alone) and reported that after 10 weeks of supplementation, vitamin E levels rose from 6.1 to 9.7 μg/mL, and this resulted in a significant reduction in PAI-1 antigen and activity, with no significant changes in the level of tPA. Our study supports the results of Bonfigli et al. (30) and goes further in showing that with a larger sample size, reduction in PAI-1 levels with AT was seen in both type 2 diabetic patients with and without MVCs.

Increasing evidence supports the role of plasma levels of soluble CAMs as emerging molecular markers of atherosclerosis (15–17). P-selectin (GMP-140) is a glycoprotein that mediates adhesion of platelets to endothelial cells during inflammation and thrombosis (18,19). Increased levels of plasma P-selectin have been observed in several vascular diseases (31–36). Furthermore, because P-selectin levels are elevated in thrombocytosis and decrease when thrombopoiesis is depressed, there is more acceptance for the concept that sP-selectin is a platelet marker rather than an endothelial cell marker (18,19). Also, Blann et al. (18,19) found no correlation between von Willebrand factor, a marker of endothelial function, and levels of soluble P-selectin, but they did observe a relationship between P-selectin levels and β-thromboglobulin, a marker of platelet activation. Furthermore, in our previous report (24), the type 2 diabetic patients did not have significantly elevated levels of E-selectin, which primarily arises from the activated endothelium. Thus, the increased P-selectin observed in our patients, especially type 2 diabetic patients with MVC,

Table 2—Baseline levels of soluble P-selectin and PAI-1

<table>
<thead>
<tr>
<th></th>
<th>Control subjects</th>
<th>Type 2 diabetic patients</th>
<th>Type 2 diabetic patients with MVCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>25</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td>P-selectin (ng/ml)</td>
<td>33.8 (22.3, 52.7)</td>
<td>57.3 (30.2, 75.9)</td>
<td>60.8 (45.2, 86)*</td>
</tr>
<tr>
<td>PAI-1 (ng/ml)</td>
<td>38.6 ± 11.5</td>
<td>45.1 ± 9.6*</td>
<td>57.7 ± 22.9*</td>
</tr>
</tbody>
</table>

Data are median (25th and 75th percentiles) for P-selectin and mean ± SD for PAI-1. *P < 0.02 significantly different compared with control subjects.
supports the view that it is largely the result of platelet hyperactivity rather than endothelial dysfunction. Skyrme-Jones and Meredith (37), in a study of 41 uncomplicated type 1 diabetic subjects, showed that relatively early in the course of type 1 diabetes, P-selectin levels are not elevated compared with matched control subjects. This could be one reason for our observation that there was a nonsignificant increase in the P-selectin levels in our type 2 diabetic patients, whereas type 2 diabetic patients with MVC had significantly elevated levels of P-selectin compared with control subjects.

AT therapy (600 IU/day for 2 weeks) has been shown to significantly reduce levels of soluble P-selectin in hypercholesterolemic patients (20). However, there is little data on the effect of AT supplementation on P-selectin levels in patients with type 2 diabetes. In our study, although soluble P-selectin levels were higher in both diabetic groups, they were significantly higher only in the diabetic group with MVCs. The majority of our patients were not hypercholesterolemic (only 19% had LDL cholesterol >130 mg/dl). We have reported previously that this latter group has significantly greater levels of high-sensitivity CRP, a prototypic marker of inflammation (23), and increased levels of urinary F2-isoprostanes, an in vivo marker of oxidative stress, compared with the group of type 2 diabetic patients without macrovascular disease (26), and it is possible that activation of P-selectin is therefore higher in type 2 diabetic patients with MVCs. In our study, as shown in hypercholesterolemic subjects in whom administration of AT (600 mg/day of AT acetate for 2 weeks) resulted in a 40% reduction in P-selectin (20), we report an average 56% reduction in P-selectin levels in all the three groups, suggesting a dose-response effect, i.e., AT may have resulted in greater reductions because of the higher doses used. There was no significant difference in the percent reduction in P-selectin among the three groups. Thus, this study goes further in demonstrating that AT decreases P-selectin levels in both reference subjects and in patients with type 2 diabetes with and without macrovascular disease.

In conclusion, we have shown that type 2 diabetes is associated with decreased fibrinolytic activity, as evidenced by increased PAI-1 and platelet activation, as evidenced by increased P-selectin. AT supplementation, in addition to decreasing LDL oxidative susceptibility, has also been shown to be anti-atherogenic, as evidenced by decreased platelet aggregation, improved endothelial function, and decreased monocyte proatherogenic activity and inflammation. In normal healthy subjects and in type 1 diabetic...
subjects, vitamin E supplementation (>300 mg/day and 1 g/day, respectively) has been shown to significantly decrease platelet aggregation (38–40). Also, in type 1 and type 2 diabetic subjects, AT (>100 IU/day) has been shown to significantly lower a potent inducer of platelet aggregation, thromboxane B2 (41,42). In regard to diabetic subjects, the data on AT therapy and LDL oxidizability is conflicting. Astley et al. (43), using 400 IU/day AT, did not show any significant effects on LDL oxidizability; however, we have shown that both LDL oxidizability and F2-isoprostanes, a marker of in vivo lipid peroxidation, is significantly decreased after AT therapy (1,200 IU/day) (24,37). Also, in type 2 diabetes, only higher doses of AT (>800 IU/day) have been shown to be anti-inflammatory, i.e., produced a significant reduction in CRP, the prototypic marker of inflammation (23). Thus, the dose of AT that was used in the Heart Outcomes Prevention Evaluation (HOPE) study (400 IU/day) (44), in which 38% of the subjects were diabetic, has not been shown to have any anti-inflammatory effects in type 2 diabetic patients. In fact, in a recent report, we showed that AT supplementation (400 IU/day) has no major anti-inflammatory effect in normal volunteers (45). Although the mechanisms by which AT reduces PAI-1 and sP-selectin levels in type 2 diabetic patients were unclear because they did not correlate to a reduction in biomarkers of oxidative stress and inflammation in our study, this will be explored in future studies.

Here, we report that therapy with high-dose AT significantly decreases both PAI-1 and P-selectin levels, and these results, if confirmed in randomized placebo-controlled clinical trials, provide evidence that AT may reduce the risk of cardiovascular events in populations with increased coronary risk. Also, dose–response studies need to be performed to define the threshold dose of AT that is anti-inflammatory. This may serve as adjunctive therapy for patients with type 2 diabetes in addition to improving glycemia and dyslipidemia and normalizing hypertension (46).

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References

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