Effects of Vitamin E Supplementation on F₂-Isoprostane and Thromboxane Biosynthesis in Healthy Cigarette Smokers

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Background—Increased formation of 8-iso-prostaglandin (PG) F₂, and thromboxane (TX) A₂, potent agonists of platelet and vascular thromboxane (TH)/PGH₂ receptors, has been detected in cigarette smokers. We performed a randomized, double-blind, placebo-controlled study of the effects of vitamin E (300, 600, and 1200 mg/d, each dose for 3 consecutive weeks) on 8-iso-PGF₂α and TXA₂ biosynthesis in 46 moderate cigarette smokers.

Methods and Results—Urinary immunoreactive 8-iso-PGF₂α and 11-dehydro-TXB₂, plasma vitamin E, and serum TXB₂ were measured by previously validated techniques. Baseline urinary 8-iso-PGF₂α and 11-dehydro-TXB₂ excretion averaged 241±78 and 430±293 pg/mg creatinine, respectively. Urinary 8-iso-PGF₂α was significantly correlated with 11-dehydro-TXB₂ (r=0.360, n=138, P<0.0001). Baseline plasma vitamin E levels averaged 20.6±4.9 μmol/L and were inversely correlated with urinary 11-dehydro-TXB₂ (r=−0.304, P=0.039) but not with 8-iso-PGF₂α (r=−0.227, P=0.129). Vitamin E supplementation caused a dose-dependent increase in its plasma levels that reached a plateau at 600 mg (42.3±11.2 μmol/L, P<0.001). This was not associated with any statistically significant change in urinary 8-iso-PGF₂α or 11-dehydro-TXB₂ excretion.

Conclusions—Supplementation with pharmacological doses of vitamin E has no detectable effects on lipid peroxidation and thromboxane biosynthesis in vivo in healthy subjects with a mild degree of oxidant stress. These findings are consistent with the hypothesis that the basal rate of lipid peroxidation is a major determinant of the response to vitamin E supplementation and have implications for the use of vitamin E in healthy subjects as well as for the design and interpretation of clinical trials of antioxidant intervention. (Circulation. 2000;102:539-545.)

Key Words: prostaglandins ■ thromboxane ■ vitamins ■ smoking ■ lipids

Vitamin E (α-tocopherol) is thought to have a role in the prevention of atherosclerosis through inhibition of oxidative modifications of LDLs. Although observational studies suggest an inverse correlation between the intake of antioxidants, such as vitamin E and β-carotene, and the incidence of coronary heart disease, clinical trials of antioxidant supplementation in patients with ischemic heart disease have yielded apparently conflicting results. This may reflect, at least in part, the variable dose and duration of vitamin E supplementation as well as the oxidant/antioxidant balance of the different patient populations entered into the trials. Moreover, the inconsistency of trial results probably reflects the inadequacy of traditional indices of lipid peroxidation in guiding the selection of the appropriate dose of vitamin E to be tested in humans. An novel analytical approach to quantify the antioxidant effect of vitamin E in vivo is provided by measurements of F₂-isoprostanes in plasma and urine. F₂-isoprostanes are formed nonenzymatically through free radical–catalyzed attack on esterified arachidonic acid, followed by their enzymatic release from cellular or lipoprotein phospholipids (reviewed in References 7, 8, and 9). F₂-isoprostanes circulate in plasma at low concentrations and are excreted in urine. 8-iso-Prostaglandin (PG) F₂α (also referred to as iPF₂α-III(κ)) is an abundant F₂-isoprostane formed in vivo in humans. This compound is of particular interest because it induces vasoconstriction and modulates the function of human platelets through the interaction with receptors that are distinct from but closely related to thromboxane (TX) A₂/PGH₂ receptors. Enhanced formation of F₂-isoprostanes has been reported in association with several cardiovascular risk factors, including hypercholesterolemia, diabetes mellitus, and cigarette smoking, that are characterized by increased lipid.
peroxidation in response to complex metabolic abnormalities or various constituents of cigarette smoke.

In both hypercholesterolemic and diabetic patients, 2-week supplementation with pharmacological doses of vitamin E (600 mg/d) was associated with normalization of enhanced F2-isoprostane formation. Similar findings were reported in apolipoprotein E–deficient mice with a long-term dosing regimen of vitamin E.19

In contrast, the short-term administration of vitamin E (100 and 800 IU/d for 5 days) to healthy chronic smokers failed to suppress urinary 8-iso-PGF2α excretion, and administration of vitamin C (2 g/d), alone or in combination with vitamin E, only partially reduced isoprostane excretion (by ~20% to 30%).18 Factors that may account for this apparent discrepancy include the small sample size (5 to 7 subjects), short duration of vitamin E supplementation (5 days), and lack of placebo control in this study.18 Alternatively, the different rates of lipid peroxidation associated with cigarette smoking versus hypercholesterolemia and diabetes mellitus might represent an important determinant of the variable effects of vitamin E supplementation in these settings. Resolving this apparent discrepancy might have implications for the interpretation of the results of recently completed clinical trials of antioxidant intervention, such as GISSI-Prevenzione4 and the Heart Outcomes Prevention Evaluation Study.5 Thus, we designed a randomized, placebo-controlled, double-blind dose-finding study of the effects of vitamin E supplementation on lipid peroxidation in moderate cigarette smokers with adequate statistical power to detect a small effect on urinary 8-iso-PGF2α excretion and sufficient duration to achieve steady-state plasma levels of vitamin E. Moreover, we examined the rate of TXA2 biosynthesis in vivo as a biological readout of changes in F2-isoprostane formation associated with vitamin E supplementation.

**Methods**

**Clinical Study Design**

Forty-eight apparently healthy volunteers (30 male and 18 female subjects, 20 to 47 years old) who had smoked 15 to 30 cigarettes per day during the previous 2 years were recruited to participate in a randomized, double-blind, placebo-controlled trial of vitamin E (d,l-α-tocopheryl acetate; Bayer SpA) supplementation. None of the subjects were taking any medication, including vitamins. During the first week of the study, the 48 smokers were screened for the inclusion and exclusion criteria of the study; during the following 3 weeks, repeated blood and urinary samples were collected for evaluation of the reproducibility of urinary 8-iso-PGF2α and plasma vitamin E levels; then, subjects were randomized to receive placebo or vitamin E 300, 600, or 1200 mg/d for 3 consecutive weeks; after completion of the treatment period, there was a washout period of 3 weeks to assess the time course of recovery. Two volunteers dropped out after the first week of treatment because of the onset of viral infection and were not replaced. Peripheral venous blood and urinary samples were collected weekly throughout the 9-week duration of the study. Urinary excretion of 8-iso-PGF2α was the primary end point of the study. We assessed compliance with the study medication as well as achievement of steady state through repeated measurement of vitamin E in plasma. To control for variable exposure to cigarette smoking among the 4 treatment groups at baseline and during the randomized phase of the study, we measured the urinary excretion of cotinine, a nicotine metabolite, weekly. We also evaluated the effects of vitamin E supplementation on the biosynthesis of TXA2 ex vivo20 and in vivo21 to explore the relationship between oxidant stress and platelet activation in the setting of chronic cigarette smoking. The protocol of the study was approved by the institutional review committees of the medical centers involved, and informed consent was obtained from all subjects.

Peripheral blood samples were drawn at 10 AM, and 1-mL aliquots were immediately transferred into glass tubes and allowed to clot at 37°C for 1 hour. Serum was separated by centrifugation and kept at ~30°C until assayed for TXB2 as a reflection of maximally stimulated cyclooxygenase activity of platelet PGH synthase-1 by endogenously formed thrombin.20 Eight-hour urine samples (~11 PM to 7 AM) were collected, the timing and total volume were recorded, and two 50-mL aliquots were stored at ~70°C until extraction. To prevent the formation of 8-iso-PGF2α in vitro, 1 mmol/L of the antioxidant 4-hydroxy-Tempo (Sigma Chemical Co) was added to 1 aliquot of each urine sample.

**Analyses of Eicosanoids and F2-Isoprostanes**

Immuno-reactive serum TXB2 and urinary 8-iso-PGF2α and 11-dehydro-TXB2 were measured by previously validated radioimmunoassay techniques.20–23 All urinary measurements were corrected for recovery and creatinine excretion.

**Other Biochemical Analyses**

Cotinine was measured by radioimmunoassay (Diagnostic Products Corp) after extraction from 10-mL aliquots of each urine sample on Sep-Pak C18 cartridges (Waters Associates) and elution with chloroform/isopropanol (85/15, vol/vol).23 Plasma vitamin E was measured by reverse-phase high-performance liquid chromatography.25

**Statistical Analysis**

With a sample size of 12 subjects per arm, the study had an 85% power (1−β) to detect a 35% difference in urinary 8-iso-PGF2α between placebo and vitamin E, with α=0.05. Statistical comparisons were made by ANOVA, and significant differences between treatments were determined by the Student-Newman-Keuls test. The Pearson coefficient (r) was calculated to quantify the direction and magnitude of correlation between variables, and linear regression was used to find the line that best predicts y from x. All data were expressed as mean±SD. A probability value of P<0.05 was assumed to be statistically significant.

**Results**

The baseline measurements obtained in the 46 healthy cigarette smokers who completed the study are detailed in the Table. Only minor differences were noted in blood lipid levels among the different groups.

Urinary cotinine excretion, a sensitive marker of exposure to cigarette smoking,26 averaged 2394±1454 ng/mg creatinine (n=138) throughout the study and was comparable in the 4 treatment groups at baseline. As shown in Figure 1, the administration of vitamin E did not affect the urinary excretion of cotinine to any statistically significant extent, nor was any appreciable time-related change measured in the placebo arm of the study.

Baseline plasma levels of vitamin E averaged 20.6±4.9 μmol/L (n=46) and were comparable in the 4 treatment groups (Table). As shown in Figure 2, vitamin E supplementation was associated with dose-dependent increases in its plasma levels that reached a plateau at 600 mg (placebo, 21.9±7.7 μmol/L; and vitamin E 300 mg/d, 33±9.2 μmol/L; 600 mg/d, 42.3±11.2 μmol/L; and 1200 mg/d, 42.7±15.3 μmol/L; P<0.001 versus baseline and versus placebo) and returned to pretreatment levels during the washout period of the study. A comparison of vitamin E
concentrations measured repeatedly during the 3 weeks of supplementation clearly demonstrates achievement of steady-state plasma levels during the treatment period of the study (Figure 2).

Baseline urinary 8-iso-PGF$_{2\alpha}$ averaged 241±78 pg/mg creatinine (n=46) and was not significantly different among the 4 treatment groups (Table). In the placebo-treated subjects, urinary 8-iso-PGF$_{2\alpha}$ excretion, assessed throughout the 9 weeks of the study, averaged 233±130 pg/mg creatinine (n=107) (intrasubject coefficient of variation, 33±11%, n=12). No statistically significant correlation was found between the excretion rate of 8-iso-PGF$_{2\alpha}$ and plasma levels of vitamin E evaluated at baseline (r = −0.227, n=46, P=0.129; Figure 3A). Increased availability of vitamin E, produced by supplementation with 300, 600, and 1200 mg/d for 3 weeks, was not associated with any statistically significant change in urinary 8-iso-PGF$_{2\alpha}$ excretion (Figure 4). The urinary excretion of 8-iso-PGF$_{2\alpha}$ and plasma levels of vitamin E measured throughout the 9 weeks of the study showed no statistically significant correlation (r = −0.065, n=138, P=0.452).

We also evaluated the potential impact of vitamin E supplementation on the maximal biosynthetic capacity of circulating platelets, as reflected by measurements of TXB$_2$ production during whole-blood clotting, 20 as well as on the actual rate of TXA$_2$ biosynthesis in vivo, as reflected by the urinary excretion of its major metabolite, 11-dehydro-TXB$_2$.21 Serum TXB$_2$ averaged 564±128 ng/mL (n=46) at baseline and was not affected by supplementation with vitamin E up to 1200 mg/d to any statistically significant extent (data not shown). Urinary 11-dehydro-TXB$_2$ averaged 430±293 pg/mg creatinine (n=46) at baseline. As shown in Figure 3B, urinary 11-dehydro-TXB$_2$ excretion rates correlated inversely with plasma vitamin E levels (r = −0.304, n=46, P=0.039). A trend for baseline urinary 11-dehydro-TXB$_2$ to correlate with urinary 8-iso-PGF$_{2\alpha}$ was apparent, but this relationship failed to attain conventional statistical significance (r = 0.285, n=46, P=0.054; Figure 5A). However,
the Pearson correlation coefficient for urinary excretion rates of 11-dehydro-TXB₂ and 8-iso-PGF₂α, measured throughout the 9 weeks of the study, was statistically significant \( (r = 0.360, n = 138, P < 0.0001; \text{Figure 5B}) \). 11-dehydro-TXB₂ excretion remained substantially unchanged during vitamin E supplementation (Figure 6).

**Discussion**

The measurement of F₂-isoprostane formation in vivo is currently accepted as a useful tool for identifying populations that may have enhanced rates of lipid peroxidation.27 In fact, elevated levels of F₂-isoprostanes have been observed in plasma and urine of animals and humans under a wide variety of conditions of enhanced oxidative stress.14–19 The finding of increased levels of F₂-isoprostanes both in the circulation and in the urine of persons who smoke17,18 is consistent with the notion that cigarette smoke contains a large number of oxidants and free radicals that could directly initiate and propagate the process of lipid peroxidation.28

As reported by Reilly et al,18 a dose-response relationship exists between the number of cigarettes smoked and the urinary excretion of 8-iso-PGF₂α. Thus, in moderate (15 to 30 cigarettes per day) and heavy (>30 cigarettes per day) smokers, the urinary excretion of 8-iso-PGF₂α averaged ≈290 and 550 pg/mg creatinine, respectively. In the moderate chronic smokers participating in the present study, the urinary

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**Figure 2.** Effects of vitamin E supplementation on plasma levels of vitamin E in healthy cigarette smokers. Plasma vitamin E was measured weekly before, during, and after treatment at same time points as in Figure 1. **Statistically significant changes (P<0.01) vs placebo and baseline. Dots and error bars represent mean±SEM values.**

**Figure 3.** Correlation between individual plasma levels of vitamin E and urinary excretion rates of 8-iso-PGF₂α (A) and 11-dehydro-TXB₂ (B) measured in 46 healthy cigarette smokers at baseline. Broken lines represent 95% CI of regression line.

**Figure 4.** Effects of vitamin E supplementation on urinary excretion of 8-iso-PGF₂α in healthy cigarette smokers. Same type of representation as used in Figure 1 is depicted for mean±SEM values measured in 4 treatment groups throughout study.

**Figure 5.** Correlation between individual urinary 8-iso-PGF₂α and 11-dehydro-TXB₂ excretion rates assessed in 46 healthy cigarette smokers at baseline (A) and throughout whole study (B). Broken lines represent 95% CI of regression line.
Although high concentrations of lipid peroxidation products were found in cigarette smokers, chronic antioxidant supplementation failed to significantly affect the urinary excretion of 8-iso-PGF2\textalpha. Failure of vitamin E to suppress isoprostane biosynthesis in this study might be related to the doses administered, the small sample size, or the short duration of the study. Thus, using 8-iso-PGF2\textalpha as the primary end point of the study, we set out to investigate the time- and dose-dependence of the antioxidant effects of vitamin E supplementation in chronic cigarette smokers.

The administration of vitamin E caused a dose-dependent increase in its plasma levels that reached steady state during the 3-week treatment study. A maximum 2-fold increase was detected during the administration of 600 mg/d of vitamin E. We have previously described a similar 2-fold increase in plasma vitamin E concentrations by supplementation with 600 mg/d in patients with type II hypercholesterolemia and in patients with type II diabetes mellitus. This is in agreement with previous studies showing that large doses of supplemental vitamin E do not increase circulating vitamin E concentrations more than ~3-fold. This is probably not due to limitation of vitamin E absorption but rather to the fact that newly absorbed vitamin E in part replaces α-tocopherol in circulating lipoproteins. In fact, >50% of the variation in plasma α-tocopherol is explained by plasma cholesterol and triacylglycerol concentrations.

Supplementation with pharmacological doses of vitamin E up to 1200 mg/d was not associated with any detectable change in urinary 8-iso-PGF2\textalpha excretion in the present study. Several factors might contribute to this negative finding. These include (1) a type II error due to inadequate sample size, (2) noncompliance with the study medication, and (3) lack of specificity of the analytical signal. In fact, given the intraindividual variability of urinary 8-iso-PGF2\textalpha excretion on repeated sampling, our study had 85% power to detect a 35% change in this biochemical end point with \( \alpha = 0.05 \). Detection of a smaller change in F2-isoprostane formation would require a much larger sample size and would probably be meaningless. Noncompliance with the study medication seems unlikely in view of the consistent changes in vitamin E levels (Figure 2). The specificity of the radioimmunological measurement of 8-iso-PGF2\textalpha might be questioned. However, the assay was previously validated by comparison with gas chromatography/mass spectrometry and has been used extensively to demonstrate changes in F2-isoprostane formation in studies of similar sample size and duration in the setting of hypercholesterolemia, non–insulin-dependent diabetes mellitus, and cystic fibrosis. The daily administration of 600 mg of vitamin E for 2 weeks to hypercholesterolemic and diabetic patients was associated with statistically significant reductions in 8-iso-PGF2\textalpha excretion by 58% and 37%, respectively (References 14 and 16 and Figure 7A). The same dose of vitamin E as administered to patients with cystic fibrosis significantly (by 42%) reduced the excretion rate of 8-iso-PGF2\textalpha (Figure 7A). Taken together, these data suggest that the same pharmacological dose of vitamin E may have variable antioxidant effects in different patient populations characterized by variable rates of lipid peroxidation. Moreover, the finding of a linear correlation between the basal rate of lipid peroxidation and the slope of changes in this index of lipid peroxidation as a function of changes in plasma vitamin E associated with short-term dosing with 600 mg/d in different clinical settings (Figure 7B) is consistent with the hypothesis that the basal rate of lipid peroxidation is a major determinant of the response to vitamin E supplementation.

Further evidence for unchanged levels of biologically active isoprostanes during vitamin E supplementation in the present study can be found in the lack of detectable changes in the rate of TXA2 biosynthesis in vivo. F2-isoprostanes and other biologically active isoeicosanoids can amplify the platelet response to other agonists in vitro. Consistent
with this concept, persistent changes in the rate of \( \text{F}_2 \)-isoprostane formation, as detected in patients with metabolic disorders,\textsuperscript{14,16} are associated with concordant changes in the rate of \( \text{TXA}_2 \) biosynthesis in vivo. Thus, we have suggested that \( \text{F}_2 \)-isoprostane formation may provide an important biochemical link between oxidant stress and platelet activation in these settings.\textsuperscript{14,16} A similar link is apparent in the present study, although the correlation between urinary excretion rates of \( \text{8-iso-PGF} \text{a} \) and \( \text{11-dehydro-TXB}_2 \) is much weaker (Figure 5) than in earlier studies of hypercholesterolemia\textsuperscript{14} and diabetes mellitus.\textsuperscript{16} The failure of vitamin E supplementation to reduce thromboxane metabolite excretion in healthy cigarette smokers in the present study provides important support to our contention\textsuperscript{14,16} that vitamin E may blunt \( \text{F}_2 \)-isoprostane–mediated amplification of platelet activation in other clinical settings, such as those depicted in Figure 7, rather than exerting a direct antiplatelet effect.

The overall picture emerging from a series of studies,\textsuperscript{14,16,18,19} as well as the present study, of vitamin E supplementation using the in vivo formation of \( \text{F}_2 \)-isoprostanes as the primary biochemical end point suggests that the effect of vitamin E on lipid peroxidation cannot be equated to that of a conventional drug blocking an enzyme or receptor in a reproducible fashion in the vast majority of patients exposed to treatment. Most likely, both the mechanisms(s) responsible for enhanced oxidant stress and the rate of lipid peroxidation are important determinants of the antioxidant effects of vitamin E supplementation. This hypothesis may help in interpreting the conflicting and largely disappointing results of recently completed trials of vitamin E supplementation in patients with ischemic heart disease.\textsuperscript{3–5}

Any protective effect of antioxidant intervention that is readily apparent in the setting of genetically determined enhanced lipid peroxidation\textsuperscript{19} is likely to be diluted by inclusion of a large proportion of patients with low levels of lipid peroxidation because of dietary habits (such as in the GISSI-Prevenzione Study, carried out in the setting of a largely Mediterranean diet\textsuperscript{4}) or lack of metabolic abnormalities associated with oxidant stress.

In conclusion, the present findings may have practical implications for the use of vitamin E supplements for cardiovascular prevention in the general population. Moreover, they suggest the need to reevaluate the response of potential target populations based on noninvasive measurements of \( \text{F}_2 \)-isoprostane formation as a rational basis for a new trial design.

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