Effects of Vitamin E on Lipid Peroxidation in Healthy Persons

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Context Oxidative stress may play a role in the development or exacerbation of many common diseases. However, results of prospective controlled trials of the effects of antioxidants such as vitamin E are contradictory.

Objective To assess the effects of supplemental vitamin E on lipid peroxidation in vivo in healthy adults.

Design Randomized, double-blind, placebo-controlled trial conducted March 1999 to June 2000.

Setting A general clinical research center in a tertiary referral academic medical center.

Participants Thirty healthy men and women aged 18 to 60 years.

Interventions Participants were randomly assigned to receive placebo or α-tocopherol dosages of 200, 400, 800, 1200, or 2000 IU/d for 8 weeks (n=5 in each group), followed by an 8-week washout period.

Main Outcome Measures Three indices of lipid peroxidation, urinary 4-hydroxynonenal (4-HNE) and 2 isoprostanes, $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI, measured by gas chromatography/mass spectrometry and compared among the 6 groups at baseline, 2, 4, 6, and 8 weeks, and 1, 3, and 8 weeks after discontinuation.

Results Circulating vitamin E levels increased in a dose-dependent manner during the study. No significant effect of vitamin E on levels of urinary 4-HNE or either isoprostane was observed. Mean (SEM) baseline vs week 8 levels of $iPF_{2\alpha}$-III were 154 (20.1) vs 168 (22.3) pg/mg of creatinine for subjects taking placebo; 165 (19.6) vs 234 (30.1) pg/mg for those taking 200 IU/d of vitamin E; and 195 (26.7) vs 213 (40.6) pg/mg for subjects taking 2000 IU/d. Corresponding $iPF_{2\alpha}$-VI levels were 1.43 (0.6) vs 1.62 (0.4) ng/mg of creatinine for subjects taking placebo; 1.64 (0.3) vs 1.24 (0.8) ng/mg for those taking 200 IU/d of vitamin E; and 1.83 (0.3) vs 1.94 (0.9) ng/mg for those taking 2000 IU/d. Baseline vs week 8 levels of 4-HNE were 0.5 (0.04) vs 0.4 (0.05) ng/mg of creatinine for subjects taking placebo; 0.4 (0.06) vs 0.5 (0.02) ng/mg with 200 IU/d of vitamin E; and 0.2 (0.02) vs 0.2 (0.1) ng/mg with 2000 IU/d.

Conclusions Our results question the rationale for vitamin E supplementation in healthy individuals. Specific quantitative indices of oxidative stress in vivo should be considered as entry criteria and for dose selection in clinical trials of antioxidant drugs and vitamins in human disease.

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OXIDATIVE STRESS APPEARS TO be of fundamental relevance to diseases as diverse as atherosclerosis, cancer, and Alzheimer disease.[1-3] However, prospective, controlled clinical trials of antioxidants present a confused picture. For example, while administration of vitamin E appeared to benefit patients with coronary disease in the CHAOS study,[4] the HOPE and GISSI Prevenzione studies failed to detect such a benefit.[5,6] Similarly, while dietary flavonoids appear to reduce cardiovascular mortality, supplemental β-carotene and vitamin A appear to increase the risk of death from lung cancer and heart disease in smokers and workers exposed to asbestos.[7,8] Various possibilities have been advanced to explain this discrepancy, including differences in patient characteristics, the antioxidant content of their diets, dose selection, and random distribution of outcomes about the mean. However, a striking feature of these and other trials of antioxidants is the absence of a biochemical basis for patient inclusion or dose selection.

Elaborate and diversified antioxidant mechanisms protect tissues from oxidative damage in humans and other organisms,[9-11] and susceptibility to benefit from exogenous antioxidants in vitro is conditioned by the degree of their depletion.[12-14] Additionally, certain vitamins with antioxidant properties can function as prooxidants, at least in vitro.[15,16] Despite these observations, nothing is known about the susceptibility of clinical trial patients to supplementation with antioxidants or whether the doses selected exhibit antioxidant effects. What little is known of the dose-response relationships of antioxidants in humans is based on an ex vivo assay of the oxidizability of low-density lipoprotein (LDL) cholesterol. Small studies using this approach report variable effects of vitamin E supplementation.[17,18] However, this assay bears an uncertain relationship to actual oxidation of LDL in vivo, and its relationship to oxida-

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tion of other lipid substances and to disease pathogenesis is unclear.

Reliable, quantitative indices of free radical–induced modification of lipids, proteins, and DNA in vivo have begun to emerge only recently.[2,19] Most information acquired in humans relates to lipid peroxidation. F_2 isoprostanes are free radical–catalyzed prostaglandin F_2 isomers.[20,21] They are chemically stable and can be measured noninvasively in urine with precision and sensitivity using homologous internal standards and mass spectrometry.[22-25] We used this approach to investigate the dose-response relationships of vitamin E with lipid peroxidation in healthy volunteers.

METHODS

Participants and Design

To investigate the dose-response relationship of vitamin E with lipid peroxidation in healthy volunteers, we performed a randomized, double-blind, placebo-controlled study from March 1999 to June 2000. The study was approved by the institutional review board of the University of Pennsylvania and the General Clinical Research Center Advisory Committee and all participants provided informed consent. Thirty volunteers, 15 men and 15 women, between the ages of 18 and 60 years (mean [SD], 38 [12.5] years) were randomized to 1 of 6 dosing groups: placebo or vitamin E at 200, 400, 800, 1200, or 2000 IU/d for 8 weeks, followed by an 8-week washout period. There were 5 subjects in each group. All volunteers were nonsmokers. In addition, they were all less than 120% of ideal body weight and had normal levels of vitamin E, vitamin C, selenium, and cholesterol at screening. Exclusion criteria included intake of any vitamin supplements within the preceding month, any medical illness, or use of any medications known to interfere with lipid metabolism within the last month. Participants were screened for study eligibility in the General Clinical Research Center at the University of Pennsylvania.

Vitamin E was supplied as (d) α-tocopherol capsules. Placebo capsules were identical in size, shape, and color. All subjects were available for measurement at all study points. Compliance was monitored by regular capsule counts and serum vitamin E levels. Twenty-four-hour urine collections for isoprostanes and serum vitamin E, vitamin C, and selenium levels were measured at baseline and at 2, 4, 6, and 8 weeks of dosing and 1, 3, and 8 weeks after dosing.

Isoprostane Measurements

Urinary $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI were assayed by stable-isotope dilution gas chromatography/mass spectrometry as previously described.[22,23] Urinary creatinine was determined using a standard automated colorimetric assay (Beckman Synchron CX System, Beckman Instruments, Arlington Heights, Ill).

Analysis of 4-Hydroxynonenal

The method for urinary measurement of 4-hydroxynonenal (4-HNE)[26] was adapted from one previously developed[27] to measure 4-hydroxyalkenals in oxidized LDL. Briefly, 5-mL urine samples were spiked with 5 ng of d3-HNE, mixed well, and allowed to equilibrate for 15 minutes at room temperature. Two milligrams of (2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride was added to each sample, and they were allowed to stand for 30 minutes at room temperature. 4-Hydroxynonenal was extracted using reverse-phase solid-extraction cartridges (C18 EC, 500 mg; International Sorbent Technology Ltd, Mid Glamorgan, Wales) under the following conditions. The cartridge was conditioned with 5 mL of ethanol and washed with 1.5 mL of distilled water. A sample was loaded onto the cartridge, which was washed with 3 mL of 60% ethanol. The cartridge was dried for 10 minutes, and the sample was eluted with 3 mL of ethyl acetate. The sample was then dried under a stream of nitrogen and dissolved in 1 mL of hexane. A second extraction used straight-phase solid-extraction cartridges (100 mg of silica conditioned with 1 mL of hexane). The sample was eluted with 1 mL of 30% ethyl acetate in hexane, dried, and dissolved in 15 µL of dodecane. One microliter of the sample was used for gas chromatography/mass spectrometry analysis. The mass spectrometer was operated in the negative-ion, electron capture ionization mode, using ammonia as the moderating gas. Ions monitored were of mass-to-mass charge ratio 283 and 286 for 4-HNE and d3-HNE, respectively.

Serum levels of vitamin E and plasma concentrations of vitamin C were measured by high-performance liquid chromatography.[28,29] Serum selenium concentrations were measured by atomic absorption spectrometry.[30]

Statistical Analysis

Tests of statistical hypothesis for main effects were based on a fixed type I error rate of 5%. The study was powered to detect a 10% change in urinary isoprostane and 4-HNE measurements. Data were initially subjected to analysis of variance with subsequent pairwise analysis using a 2-tailed *t* test, as appropriate. Data are expressed as mean (SEM). To account for potential differences among group baseline values, all successive time values were adjusted for each subject's baseline measure: $\Delta(i,k) = \text{value}(i,k) - \text{value}(i,0)$, for each subject i at $k = 2$, 4, 8, 9, 12, and 16 weeks. These adjusted measurements were subjected to an analysis of variance appropriate for a 2-factor experiment design with 1 repeated measure (time) and 1 nonrepeated measure (dose).

RESULTS

Prior to dosing, all measurements of endogenous antioxidants were within normal limits. Serum vitamin E increased in a dose-dependent manner and reached a steady state by 8 weeks. For example, levels at baseline vs 8 weeks of placebo were 9.4 (2.1) vs 8.5 (0.8) mg/dL (reference range for serum vitamin E, 4.6-14.5 mg/dL). Levels rose from 8.4 (1.4) to 20.7 (2.8) mg/dL in subjects taking 200 IU/d and from 8.9 (1.0) to 52.8 (4.7) mg/dL in those taking 2000 IU/d. Vitamin E levels declined to preintervention levels at 8 weeks following dosing (week 16). Levels of endogenous selenium and vitamin C were unaltered by vitamin E supplementation.

Urinary isoprostanes were also unaltered by vitamin E therapy. For example, the corresponding baseline vs week 8 levels of urinary $iPF_{2\alpha}$-III were 154 (20.1) vs 168 (22.3) pg/mg of creatinine for subjects taking placebo; 165 (19.6) vs 234 (30.1) pg/mg of creatinine for those taking 200 IU/d of vitamin E; and 195 (26.7) vs 213 (40.6) pg/mg of creatinine for subjects taking 2000 IU/d (FIGURE, A). None of these changes attained statistical significance. Similarly, urinary $iPF_{2\alpha}$-VI levels were 1.43 (0.6) vs 1.62 (0.4) ng/mg of creatinine for subjects taking placebo; 1.64 (0.3) vs 1.24 (0.8) ng/mg of creatinine for those taking 200 IU/d of vitamin E; and 1.83 (0.3) vs 1.94 (0.9) ng/mg of creatinine for those taking 2000 IU/d (Figure, B). None of these differences were statistically significant.

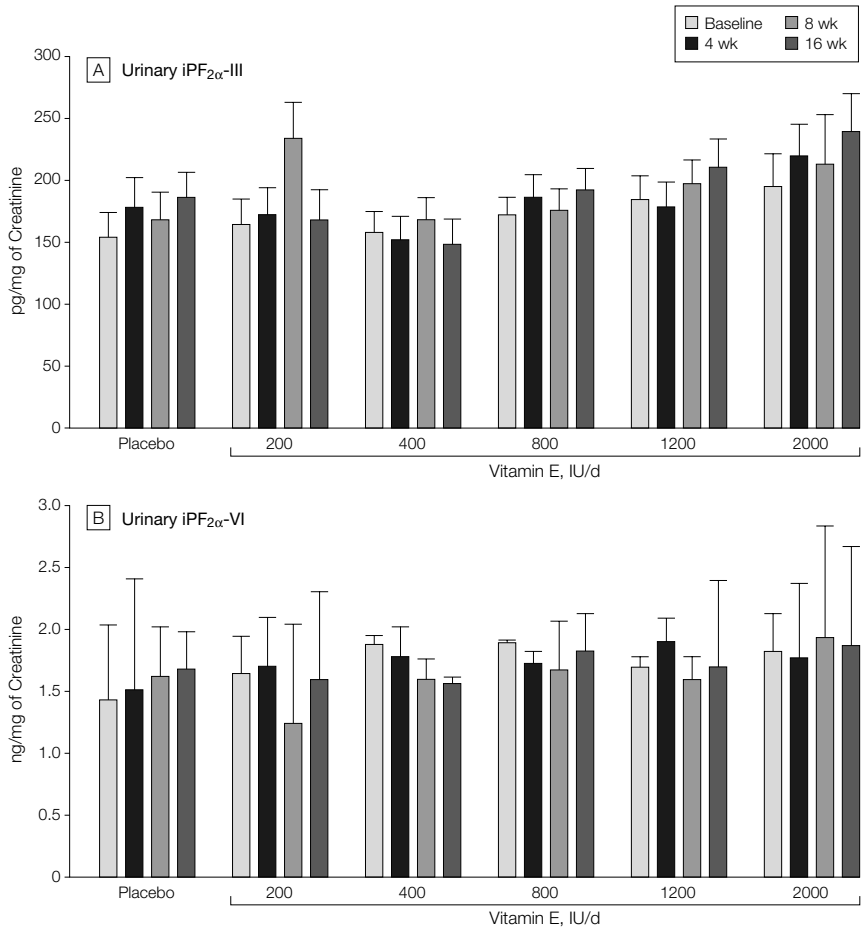
Urinary 4-HNE was also unaltered by vitamin E supplementation. For example, levels were 0.5 (0.04) vs 0.4 (0.05) ng/mg of creatinine for subjects taking placebo; 0.4 (0.06) vs 0.5 (0.02) ng/mg of creatinine after 8 weeks of therapy with 200 IU/d of vitamin E; and 0.2 (0.02) vs 0.2 (0.10) ng/mg of creatinine after 8 weeks of 2000 IU/d of vitamin E. No adverse reactions to vitamin E supplementation were reported.

The repeated measures design of this study enabled adjustment for baseline variation among the dosing groups. No effect was seen following subtraction of predosing baseline values for each of the variables of interest. For example, the absolute changes in $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-VI, and 4-HNE excretion after 8 weeks of 800 IU/d of vitamin E were –2.76 (5.35) pg/mg of creatinine, –0.01 (0.27) ng/mg of creatinine, and 0.10 (0.05) ng/mg of creatinine, respectively.

Given a nonsignificant ($P=.13$) second-order interaction between dose and time for 4-HNE, we examined the main effects, dose and time, which were each statistically nonsignificant ($P=.71$ and $P=.13$, respectively). The second-order interaction (dose × time) for $iPF_{2\alpha}$-III also failed to attain significance ($P=.62$). Dose and time were again statistically nonsignificant ($P=.31$ and $P=.14$, respectively). Finally, the second-order interaction (dose × time) for $iPF_{2\alpha}$-VI was also nonsignificant ($P=.92$). Dose and time were also statistically nonsignificant ($P=.49$ and $P=.25$, respectively).

Given that studies of LDL oxidation ex vivo[17,18] have suggested that the effects of vitamin E supplementation approach a maximum at daily doses of 400 IU, we performed a subsidiary analysis that compared the effects of the combined doses of 400, 800, 1200, and 2000 IU/d with placebo. We performed *t* tests for independent samples for 4-HNE, $iPF_{2\alpha}$-III, and $iPF_{2\alpha}$-VI to compare the 5 participants who received placebo with the 20 who received ≥400 IU/d of vitamin E. In all cases, nonsignificant *P* values were obtained: .78, .84, and .52 respectively.

Figure. Urinary Isoprostanes $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI Stratified by Vitamin E Intake



Data represent the mean (SEM) measurements for each group.

COMMENT

Oxidative damage is widely implicated in the pathogenesis of disease. While traditional indices of this process in integrated systems are recognized as fallible, several novel approaches have been developed that permit quantitation of the consequences of excessive free radical generation in vivo. Isoprostanes are free radical–catalyzed isomers of prostaglandins, peroxidation products of arachidonic acid.[20,21] Initially formed in situ in the phospholipid domain of cell membranes, they are cleaved out, circulate, and are excreted in urine. They effect a range of biological activities in vitro and may activate both membrane receptors for traditional prostaglandins[31] and peroxisomal proliferator activated receptors in the nucleus.[32] Given the complexity of the species (up to 64 F_2 isoprostanes

can be generated), we have developed highly specific assays for individual isoprostanes rather than using semiquantitative estimates of "total isoprostanes."[21] Using this approach, we have demonstrated increased generation of isoprostanes in cigarette smokers,[33] abusers of alcohol,[34] and in persons with a range of ischemia/reperfusion[25,35,36] and inflammatory syndromes.[37-40]

In the present study, we sought an effect of vitamin E on 2 isoprostanes, $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI. The former compound may be generated either by cyclooxygenase (COX) turnover or by a free radical–dependent mechanism.[22,41] However, the COX-dependent pathway contributes to an undetectable extent to urinary $iPF_{2\alpha}$-III, even in syndromes of COX activation.[33,42] By contrast, $iPF_{2\alpha}$-VI, a more abundant entity, is formed only as a product of lipid peroxidation.

Along with isoprostanes, we also measured 4-HNE, an independent index of lipid peroxidation. Like the isoprostanes, urinary 4-HNE is increased in patients with alcohol-induced liver disease. In these patients, who are deficient in endogenous vitamin C, supplements of vitamin C reduce their elevated levels of both urinary isoprostanes and 4-HNE.[34] Similarly, supplementation with vitamin C, but not vitamin E, reduces elevated levels of urinary $iPF_{2\alpha}$-III in cigarette smokers, who are also selectively depleted of vitamin C.[33] We have also shown that exogenous vitamin E alone or in combination with vitamin C reduces elevated levels of urinary isoprostanes in patients with virally induced cirrhosis,[43] the antiphospholipid syndrome,[40] and obstructive pulmonary disease.[37] Elevated urinary isoprostanes can also be used to select a rational dose of an antioxidant, such as vitamin E. For example, a dosage of vitamin E that suppresses elevated levels of urinary $iPF_{2\alpha}$-VI retards atherogenesis in the apolipoprotein E–deficient mouse,[44] whereas a lower dosage, selected without a biochemical rationale, fails to influence atherogenesis in this model.[45]

Despite these observations, we failed to detect any impact of vitamin E on 3 quantitative indices of lipid peroxidation. This was true when we examined the absolute values of these indices after adjustment for interindividual baseline variation. This was also true when we compared the values after placebo with those after ≥400 IU/d of. vitamin E, doses at which effects on LDL oxidizability ex vivo have been noted.[17,18] We administered vitamin E over a broad dose range, incorporating the doses used in the CHAOS, HOPE, and GISSI Prevenzione studies,[4-6] all considerably in excess of the recommended daily allowance.[46] We administered the natural (d) α-tocopherol isomer of vitamin E for a sufficient period (8 weeks) to achieve steady-state incorporation into cell membranes.[47-49]

The study population included individuals assessed as consuming a diet replete with endogenous vitamin E. Indices of endogenous antioxidant defense, including vitamin E, were in the normal range prior to initiation of the study. Vitamin E levels increased in a dose-dependent manner to a maximum of roughly 5-fold, thus excluding protocol noncompliance as an explanation for our results. Although the relatively small size of this study may have precluded detection of subtle effects of vitamin E, the study was designed to detect changes of at least 10% in any of the parameters.

This study has implications for the evaluation of clinical trials of antioxidants and for the widespread consumption of antioxidants by apparently healthy individuals. First, the inclusion of patients without biochemical evidence of increased oxidative stress in clinical trials of antioxidants would be expected to dilute the population susceptible to benefit, even assuming the functional importance of oxidative stress in the disease under evaluation. This might seriously undermine the sample size calculations used in such trials, leaving them open to a type II statistical error and outcomes reflecting random variation about the mean. Such issues occurred for a decade in clinical trials of aspirin in cardiovascular disease. The absence of an index of thromboxane biosynthesis led to the inclusion of many patients in whom, in retrospect, thromboxane-dependent platelet activation was unlikely to have been abnormal. This, in turn, reduced the ability to detect a significant cardioprotective effect of aspirin.[50-52] Detection of marked increases in thromboxane biosynthesis in the ischemic episodes of unstable angina and coincident with therapeutic thrombolysis[53] afforded the rationale for clinical studies that clearly demonstrated the efficacy of aspirin in these settings.[54,55]

Second, incorporation of measurements such as urinary isoprostanes and 4-HNE may also be used in the rational selection of antioxidant dosages for such trials. Several studies have indicated the prooxidant potential of antioxidant vitamins, including vitamin E,[16] and this may have functional relevance in vivo. For example, low doses of α-tocopherol improve endothelial function in hypercholesterolemic rabbits, but is worsened by higher doses of the vitamin.[56] We found no evidence of a net prooxidant effect of vitamin E. It is theoretically possible that competing prooxidant and antioxidant effects of vitamin E canceled each other out, but if so, this was unrelated to dosage. Although we and others have provided some information on the biochemical effects of such supplements in vivo in diseased populations, the optimal antioxidant regimens for specific conditions may vary.[57] Such regimens should be defined before initiating large-scale clinical trials.[58]

Finally, the average Western diet provides the recommended daily allowance of vitamin E,[46] and the endogenous levels in the individuals in our study fell within the normal range. We found no evidence of additional effects of supplementing these individuals with a range of dosages of vitamin E on their rate of lipid peroxidation in vivo. Our findings question the potential benefit of the reportedly widespread consumption of vitamin E by such healthy individuals.[59]

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Acquisition of data: Meagher, Barry, Lawson, Rokach, FitzGerald.
Analysis and interpretation of data: Meagher, Barry, FitzGerald.
Drafting of the manuscript: Meagher, Barry.
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