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Vitamin E Supplementation in Patients With Carotid Atherosclerosis

Reversal of Altered Oxidative Stress Status in Plasma but not in Plaque

Fausta Micheletta, Silvia Natoli, Maria Misuraca, Enrico Sbarigia, Ulf Diczfalusy, Luigi Iuliano

Objective—Oxidative stress is believed to play a pivotal role in the initiation and progression of atherosclerosis. We analyzed whether vitamin E supplementation influences oxidative stress in plasma and atherosclerotic plaques of patients with severe atherosclerosis.

Methods and Results—In 16 patients who were candidates for carotid endarterectomy and in 32 age- and sex-matched controls, plasma levels of 7 β -hydroxycholesterol, 7-ketocholesterol, cholesterol, and vitamin E were measured. Patients were randomly allocated to standard treatment with or without 900 mg/d vitamin E. After 6 weeks of treatment, the reported variables were measured in plasma and plaques. The plasma vitamin E/cholesterol ratio was significantly lower in patients than in controls (3.05 ± 0.6 versus 6.3 ± 1.7 $\mu\text{mol}/\text{mmol}$ cholesterol, $P < 0.001$). Plasma 7 β -hydroxycholesterol was significantly higher in patients than in controls (5.0 ± 1.04 versus 4.4 ± 0.6 ng/mL, $P < 0.05$). Patients who were given vitamin E supplementation showed a significant increase of plasma vitamin E with concomitant decrease of 7 β -hydroxycholesterol. Conversely, no treatment dependence was observed in oxysterol or vitamin E content of plaques.

Conclusions—An imbalance between oxidative stress and antioxidant status is present in patients with advanced atherosclerosis. Vitamin E supplementation improves this imbalance in plasma but not in plaques. (*Arterioscler Thromb Vasc Biol.* 2004;24:136-140.)

Key Words: atherosclerosis ■ oxidative stress ■ vitamin E ■ oxysterols ■ carotid plaques

There is considerable interest in the role of oxidative stress in several disease settings, including atherosclerosis.^{1,2} A large number of clinical trials with antioxidants, mainly vitamin E, in patients with or at risk for cardiovascular disease was performed, but in most cases no effect of vitamin E on the incidence of cardiovascular events was noted.³ Consequently, the usefulness of antioxidants in cardiovascular diseases has been a matter for debate in the scientific community.⁴ Several hypotheses have been put forward to explain the discordant results of interventional trials based on antioxidant treatment, including the compliance, design of the trials, and dosage and source of vitamin E used.^{5,6}

An important argument of debate is the striking difference of vitamin E effects in experimental and clinical studies. In contrast to clinical trials with antioxidants, there is compelling evidence in favor of the antiatherosclerotic effect of vitamin E in experimental models of atherosclerosis.⁷ One possible explanation for this discrepancy is that in experimental models, vitamin E treatment has been able to affect the early stage of atherosclerosis, whereas clinical trials enrolled patients with advanced atherosclerosis who are not

sensitive to antioxidant treatment. The demonstration that antioxidants are ineffective in advanced human atherosclerosis could help to interpret the negative results of clinical trials and also to modify the design of future trials. To specifically address this issue, we studied candidates for carotid endarterectomy, because of a critical stenosis, by measuring the circulating levels of vitamin E and oxysterols, markers of oxidative stress, in comparison to a control population. Furthermore, we measured these variables in plasma and atherosclerotic plaques, taken after endarterectomy, of patients who were randomized to standard treatment or standard treatment plus 900 mg/d vitamin E. The present study reports for the first time that in patients with advanced atherosclerosis, supplementation with vitamin E did not affect vitamin E or oxysterol content of atherosclerotic plaque.

Methods

Sixteen consecutive patients with carotid atherosclerosis having a lumen stenosis $>70\%$ and eligible for carotid endarterectomy were enrolled in the study. Exclusion criteria were acute and chronic liver disease, cancer, malabsorption syndrome, prior stomach surgery, renal failure, and the use of any supplements containing vitamin E,

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vitamin C, carotenoids, or iron in the 30 days before the study. Eligible patients were randomly assigned to take standard antithrombotic therapy with acetylsalicylic acid or ticlopidin plus 900 mg/d vitamin E (α -tocopherol, 450 IU/d) ($n=8$) or to continue antithrombotic therapy ($n=8$) for 6 weeks. This supplementation period was consistent with present clinical practice in our hospital. Thirty-two healthy volunteers matched for age and sex were enrolled as control group from the department staff and their relatives. Nobody used any supplements containing vitamin E, vitamin C, carotenoids, or iron for at least 30 days before the enrollment. All subjects gave informed consent to participate in the study.

Baseline blood samples were taken from patients and controls who had fasted for at least 12 hours, between 8:00 PM and 9:00 AM for the evaluation of plasma α -tocopherol, 7β -hydroxycholesterol, 7-ketocholesterol, total cholesterol, and triglycerides. Patient blood samples were also obtained after the treatment period on the day before endarterectomy. Atherosclerotic plaques obtained at endarterectomy were collected for the evaluation of α -tocopherol, 7β -hydroxycholesterol, 7-ketocholesterol, and cholesterol. As normal arteries, thoracic aorta segments were obtained from healthy donors undergoing transplantation surgery ($n=6$). The healthy donors were 14 to 43 years of age (mean, 32 years); all were accident victims. The study protocol was approved by the local ethical committee.

Tissue Assay

Immediately after collection, plaques and normal arteries were washed from contaminating blood with saline. All carotid endarterectomy specimens obtained were advanced lesions belonging to type V through VI according to Stary's classification.⁸ The plaque samples were cleaned from debris, put on filter paper to absorb liquid, and then weighed and stored at -80°C until assay. Aorta rings from transplant donors showed no macroscopic evidence of atherosclerosis. Intimas (and inner media) were stripped from aortic rings with the aid of microdissection tweezers, dried, weighed, and stored at -80°C until assay.

On the day of assay, tissue samples were finely minced and transferred with 10 mL chloroform/methanol (2:1, v/v) solution containing 0.01% butylated hydroxytoluene (v/w) to a glass homogenizer. Vitamin E acetate and deuterium-labeled cholesterol, 7β -hydroxycholesterol, and 7-ketocholesterol as internal standards, and butylated hydroxytoluene and EDTA as antioxidant and metal-complexing reagent, respectively, were added. Vitamin E, cholesterol, and oxysterols were measured in the same sample by a recently developed method based on high-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS).⁹ Briefly, the tissue homogenate was extracted, and the organic phase was processed for oxysterols and cholesterol determination (4.8 mL) and for vitamin E determination (0.200 mL). Vitamin E was analyzed by HPLC with α -tocopherol acetate as internal standard.¹⁰ Samples for oxysterol and cholesterol determinations were subjected to alkaline hydrolysis, pH adjustment, and re-extraction. Then, the organic phase was processed for GC/MS assay.⁹ The mass spectrometer was operated in the selected ion monitoring mode, and the ions used for analysis were as follows: [$^2\text{H}_6$]cholesterol, 335 m/z; cholesterol, 329 m/z; [$^2\text{H}_6$]7 β -hydroxycholesterol, 462 m/z; 7 β -hydroxycholesterol, 456 m/z; [$^2\text{H}_6$]7-ketocholesterol, 478 m/z; and 7-ketocholesterol, 472 m/z.¹¹

Plasma Assay

Plasma levels of vitamin E were analyzed by HPLC as previously described,¹⁰ and expressed as μmoles vitamin E/mmol cholesterol. Plasma oxysterols were measured by MS, using an isotope dilution method as previously described.⁹ Serum cholesterol and triglycerides were measured with commercial kits.

Statistical Analysis

Means and SD were calculated for all measured parameters. A Student *t* test was used to identify differences in each parameter between patients and controls. Posttreatment values were compared

with baseline values by the paired Student *t* test. A difference was considered statistically significant at $P<0.05$.

Results

The baseline characteristics of the study populations are described in Table 1. There was no difference in age, sex, smoking habit, cholesterol, and triglycerides between patients with carotid atherosclerosis and healthy subjects. Eleven patients had hypertension, seven had hypercholesterolemia, and five had diabetes. There was no difference in any variable considered between supplemented and nonsupplemented groups.

Plasma vitamin E levels were $\approx 50\%$ lower in patients affected by carotid atherosclerosis compared with controls. Plasma $7\beta\text{OH}$ -cholesterol was significantly higher in patients compared with controls. However, plasma 7-ketocholesterol concentrations in patients with carotid atherosclerosis were comparable to those of control subjects.

α -Tocopherol and oxysterols were evaluated in plasma and in plaques of patients with or without vitamin E supplementation. After 6 weeks of this treatment, plasma vitamin E levels significantly increased in the supplemented group by $\approx 88\%$ (from 3.15 ± 0.65 to 5.92 ± 1.34 $\mu\text{mol}/\text{mmol}$ cholesterol; $P<0.001$) (Figure), whereas no change was observed in patients who did not take a vitamin E supplement (data not shown). In the supplemented group, 7β -hydroxycholesterol was significantly reduced at the end of treatment period (from 4.94 ± 1.6 to 3.56 ± 1.1 ng/mL; $P=0.032$) (Figure). The 7ketocholesterol concentration in plasma was not affected by vitamin E supplementation (Figure). No changes of cholesterol and triglyceride concentrations were observed at the end of follow-up.

Data on vitamin E and oxysterols in atherosclerotic plaques and normal arteries are shown in Table 2. Higher concentrations of cholesterol oxidation products, $7\beta\text{OH}$ - and 7ketocholesterol, were observed in atherosclerotic plaques compared with normal arteries (0.27 ± 0.12 versus 0.04 ± 0.01 and 0.98 ± 0.2 versus 0.38 ± 0.2 $\mu\text{mol}/\text{mmol}$ cholesterol, respectively; $P<0.05$). As far as vitamin E in the vessel wall is concerned, the difference between carotid plaques and normal arteries was also evident (2.06 ± 0.7 versus 0.54 ± 0.3 $\mu\text{mol}/\text{mmol}$ cholesterol; $P=0.005$).

The ratio between 7β -hydroxycholesterol and vitamin E in atherosclerotic plaques was ≈ 200 times higher than that in plasma (142×10^{-3} versus 0.68×10^{-3} , respectively).

At the end of treatment period, α -tocopherol content of carotid plaques was similar in patients with or without vitamin E supplementation (Table 2). Similarly, no difference in oxysterols content was observed in the plaques from the 2 groups (Table 2).

Discussion

The controversial results of clinical trials with antioxidants in cardiovascular disease obtained in the past decade have been attributed to several factors. It has been suggested that the major determinant is the lack of data on oxidative stress status in patients with cardiovascular disease. Several methods to measure oxidative stress, including thiobarbituric acid test, xylenol orange assay, or LDL oxidability *ex vivo* have been

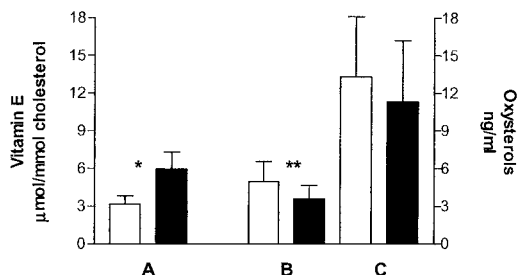
TABLE 1. Baseline Characteristics of Study Populations

	Carotid Atherosclerosis Patients (n=16)	Healthy Controls (n=32)	P
Age, y	69.6±6.6	67.8±8.3	NS
Male sex, n (%)	11 (68.7)	22(68.7)	NS
Smoking habit, n (%)	7 (43.7)	10(31)	NS
Triglycerides, mmol/L	1.55±0.9	1.67±1.1	NS
Cholesterol, mmol/L	5.9±1.2	5.83±1.1	NS
Vitamin E, $\mu\text{mol}/\text{mmol}$ cholesterol	3.05±0.6	6.3±1.7	<0.001
7 β OH-cholesterol			
ng/mL	5.0±1.04	4.4±0.6	<0.05
nmol/mmol cholesterol	2.1±0.4	1.8±0.2	<0.05
7keto-cholesterol			
ng/mL	12.7±3.7	13.6±4.9	NS
nmol/mmol cholesterol	5.4±1.5	5.8±2.1	NS
Hypercholesterolaemia, n(LDL-C \geq 3.5 mmol/L)	7	—	—
Hypertension, n	11	—	—
Diabetes, n	5	—	—
Previous transient ischemic attack or stroke, n	5	—	—
Coronary artery disease, n	5	—	—
Peripheral vascular disease, n	2	—	—

Values are mean±SD or n (%).

Hypercholesterolaemia indicates patients in treatment with statins; coronary artery disease, the presence of a previous myocardial infarction or chronic stable angina; and peripheral vascular disease, the presence of intermittent claudication and an ankle-brachial index <0.9.

used.^{12–14} These methods are suitable for simple in vitro systems but have strong analytical limitations when applied in vivo in plasma and urine owing to the complexity of these biological matrices. Recently, new markers based on the detection of lipid peroxidation products by MS, such as isoprostanes and oxysterols, have been proposed as specific and sensitive markers of oxidative stress in vivo.^{15–18} Oxysterols are cholesterol oxidation products, and among them, 7 β OH-cholesterol and 7-ketocholesterol, produced by free-radical nonenzymatic reactions, have been proposed as valuable oxidative stress markers in plasma.^{17,18} Previous studies demonstrated that 7 β OH-cholesterol is elevated in smokers compared with nonsmokers and is associated with progression of carotid atherosclerosis.^{19,20} We provided further support to these findings, inasmuch as the circulating levels of 7 β OH-cholesterol were significantly elevated in patients with advanced atherosclerosis compared with healthy subjects. In



Plasma vitamin E (A), 7 β OH-cholesterol (B) and 7keto-cholesterol (C) in patients given vitamin E supplementation at baseline (open bars) and after supplementation (filled bars). Values represent mean±SD. * $P<0.001$; ** $P<0.05$.

addition, we found a dramatically reduced concentration of α -tocopherol in plasma. This finding resembles the kinetics of LDL oxidation in vitro during which the evolution of oxidation products is preceded by consumption of constitutive antioxidants.^{21–23} In this context, although 7 β OH-cholesterol functions as a more sensitive marker of in vivo lipid peroxidation, 7-ketocholesterol is produced at a faster rate in vitro.⁹

The present study is the first that simultaneously measured the antioxidant/oxidative stress status in plasma and in human atherosclerotic plaques. Analysis of vitamin E in the plaque demonstrated high values of this vitamin, which is apparently in accordance with previous findings indicating that in atherosclerotic or aging arteries vitamin E is elevated.^{24,25} However, these studies did not simultaneously measure vitamin E in the plasma or adjust vitamin E for the cholesterol present in the vessel wall. We found that in our patients, the vitamin E/cholesterol ratio of the plaque is almost similar to that of the plasma and clearly lower than that found in peripheral circulation of healthy subjects. Furthermore, the analysis of oxysterols in plaques showed a large amount of these compounds compared with normal arteries; interestingly, the ratio between 7 β OH-cholesterol and vitamin E in atherosclerotic plaques is \approx 200 times higher than that in plasma. These data suggest that vitamin E levels are reduced in plasma and atherosclerotic plaques of patients with advanced atherosclerosis.

Patients supplemented with vitamin E showed a marked increase of circulating levels of vitamin E and a simultaneous, but less evident, decrease of 7 β OH-cholesterol, suggesting

TABLE 2. Vitamin E and Oxysterol Content in Atherosclerotic Plaques and Normal Arteries

	Atherosclerotic Plaques		Normal Vessels
	Supplemented Group	Nonsupplemented Group	
Weight, mg	287.1±99	244±54.1	268.1±37.5
Cholesterol, nmol/mg tissue	34.8±24.5	40.6±28.7	4±2.4
Vitamin E			
μmol/mmol cholesterol	2.15±1.1	2.06±0.7	0.54±0.3
pmol/mg tissue	71.7±48.3	71.3±49.2	1.65±0.4
7βOH-cholesterol			
μmol/mmol cholesterol	0.33±0.17	0.27±0.12	0.04±0.01
pmol/mg tissue	9.34±5.9	9.77±7.8	0.2±0.1
7keto-cholesterol			
μmol/mmol cholesterol	0.89±0.18	0.98±0.2	0.38±0.2
pmol/mg tissue	30.7±12.6	39.7±14	1.9±1

Values are mean±SD or n (%).

that in human circulation, vitamin E may in part influence the formation of 7βOH-cholesterol, according to previous findings.²⁶ The analysis of atherosclerotic plaques showed that vitamin E supplementation did not affect vitamin E or oxysterol content of atherosclerotic plaques. These data are apparently in disaccord with a previous experimental study in which vitamin E was shown to be taken up by arterial wall after oral supplementation.²⁷ However, in experimental models of advanced atherosclerosis no data exist to show that vitamin E can accumulate within the arterial wall.

Our data have several clinical implications. First of all, they can help in interpreting the negative results of clinical trials with vitamin E, which have been predominantly performed in patients with advanced atherosclerosis. Thus, in an experimental model vitamin E was shown to reduce isoprostanes in atherosclerotic plaques and eventually retard the progression of lesions, suggesting a causative role for oxidative stress in the pathogenesis of atherosclerosis.²⁸ In the study, however, the vitamin E content of the plaque was not measured; therefore, it was not clear if the reduction of isoprostanes in atherosclerotic lesions resulted from an inhibited formation in situ or conversely reflected the decreased levels obtained in peripheral circulation. Assuming, however, that reduced oxidative stress is a prerequisite for retarding atherosclerosis progression, vitamin E treatment should be associated with a decrease of oxidative stress within the plaques. In contrast with observations in experimental atherosclerosis, we demonstrated that in advanced atherosclerotic lesions, vitamin E supplementation did not influence the vitamin E level in the plaque. This lack of effect implicates that accumulation of vitamin E in the plaque is not regulated by simple diffusion kinetics from the plasma compartment and the need of turnover studies. These results imply that patients with advanced atherosclerosis in clinical trials with antioxidants do not represent an ideal model for testing the clinical effect of vitamin E, and antioxidant treatment should be tested in patients with early atherosclerosis. This is supported by the results of the recently published Antioxidant

Supplementation in Atherosclerosis Prevention (ASAP) study,²⁹ which showed a significant reduction of atherosclerosis progression by long-term supplementation with a combination of vitamin E and C.

An apparent limitation of the hypothesis that antioxidants are effective only in the early stages of atherosclerosis comes from a recent report by Cyrus et al³⁰ on the effect of vitamin E on experimental atherosclerosis. The investigators showed that α-tocopherol reduced progression of atherosclerosis by suppressing oxidative stress in the LDL receptor knockout mice, either at early phase of atherogenesis or after the disease is established. However, it remains to be defined whether the established lesion in this animal model is comparable to the human advanced lesion of carotids.

In conclusion, we demonstrated that patients with severe atherosclerosis have an imbalance of oxidant/antioxidant status. Vitamin E supplementation corrects the oxidant/antioxidant balance in plasma. However, in atherosclerotic plaques, supplementation did not affect vitamin E levels. Facilitation of vitamin E transport within atherosclerotic plaque may represent an important target for the treatment of early stage atherosclerosis progression.

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