See corresponding editorial on page 530.

Mixed tocopherols inhibit platelet aggregation in humans: potential mechanisms[1–3]

Meilin Liu, Agneta Wallmon, Caroline Olsson-Mortlock, Rolf Wallin, and Tom Saldeen

ABSTRACT

Background: Epidemiologic studies have shown an inverse correlation between acute coronary events and high intake of dietary vitamin E. Recent clinical studies, however, failed to show any beneficial effects of α-tocopherol on cardiovascular events. Absence of tocopherols other than α-tocopherol in the clinical studies may account for the conflicting results.

Objective: This study compared the effect of a mixed tocopherol preparation rich in γ-tocopherol with that of α-tocopherol on platelet aggregation in humans and addressed the potential mechanisms of the effect.

Design: Forty-six subjects were randomly divided into 3 groups: α-tocopherol, mixed tocopherols, and control. ADP and phorbol 12-myristate 13-acetate–induced platelet aggregation, nitric oxide (NO) release, activation of endothelial constitutive nitric-oxide synthase (ecNOS; EC 1.14.13.39) and of protein kinase C (PKC), and ecNOS, superoxide dismutase (SOD; EC 1.15.1.1), and PKC protein content in platelets were measured before and after 8 wk of administration of tocopherols.

Results: ADP-induced platelet aggregation decreased significantly in the mixed tocopherol group but not in the α-tocopherol and control groups. NO release, ecNOS activation, and SOD protein content in platelets increased in the tocopherol-treated groups. PKC activation in platelets was markedly decreased in the tocopherol-treated groups. Mixed tocopherols were more potent than α-tocopherol alone in modulating NO release and ecNOS activation but not SOD protein content or PKC activation.

Conclusions: Mixed tocopherols were more potent in preventing platelet aggregation than was α-tocopherol alone. Effects of mixed tocopherols were associated with increased NO release, ecNOS activation, and SOD protein content in platelets, which may contribute to the effect on platelet aggregation. *Am J Clin Nutr* 2003;77:700–6.

KEY WORDS Platelets, platelet aggregation, tocopherols, mixed tocopherols, α-tocopherol, nitric oxide, endothelial constitutive nitric-oxide synthase, superoxide dismutase, protein kinase C

INTRODUCTION

The results of epidemiologic studies have shown an inverse correlation between acute coronary events and high dietary intake of vitamin E (1–4). Two large clinical trials (5, 6), however, failed to show any beneficial effect of α-tocopherol on cardiovascular events and cardiac death. In the clinical studies, the vitamin E preparation contained α-tocopherol alone, whereas vitamin E in food consists of several different tocopherols. Thus, absence of tocopherols other than α-tocopherol in the preparations used in the clinical studies may account for the conflicting results. Among the other tocopherols, γ-tocopherol in particular has been shown to have potent antioxidant effects (7), and γ-tocopherol, but not α-tocopherol, is reduced in patients with coronary heart disease (8).

Platelet aggregation plays an important role in thrombosis and cardiovascular events (9–11). In an experimental investigation, we found that a preparation of mixed tocopherols rich in γ-tocopherol was more potent than α-tocopherol alone in decreasing platelet aggregation and intraarterial thrombus formation in rats (12).

The present study was designed to compare the effect of the same γ-tocopherol–rich preparation of mixed tocopherols with that of α-tocopherol alone on platelet aggregation in human subjects. We also investigated potential mechanisms underlying the effect, such as influence on nitric oxide (NO), endothelial constitutive nitric-oxide synthase (ecNOS; EC 1.14.13.39), protein kinase C (PKC), and superoxide dismutase (SOD; EC 1.15.1.1).

SUBJECTS AND METHODS

Subjects and study design

Forty-six healthy subjects aged 33–74 y ($\bar{x}$: 52.2 ± 1.5 y) were randomly assigned to 3 groups: 18 subjects were given α-tocopherol alone, 18 received mixed tocopherols, and 10 were untreated control subjects. All subjects completed the study. Compliance was good (>95%), as assessed by the number of pills returned. No subjects had used any platelet inhibitors. Exclusion criteria included heavy drinking, smoking, and taking other drugs or antioxidants (such as vitamin C) interfering with platelet function in the 2 wk before the study and during the experimental period.

Blood samples were drawn before entry into the study for assessment of basal values. During the following 8 wk, the subjects were given a daily oral dose of natural mixed tocopherols

[1] From the Department of Surgical Sciences, Section of Forensic Medicine, University of Uppsala, Sweden.

[2] Supported by grants from the Swedish Medical Research Council (to TS) and from the National Board of Forensic Medicine, Sweden (to CO-M and TS).

[3] Address reprint requests to T Saldeen, Department of Surgical Sciences, Section of Forensic Medicine, University of Uppsala, Dag Hammarskjölds väg 17, Uppsala 75 237, Sweden. E-mail: tom.saldeen@kirurgi.uu.se.

Received February 25, 2002.

Accepted for publication October 7, 2002.

The American Journal of Clinical Nutrition

(100 mg γ-, 40 mg δ-, and 20 mg α-tocopherol corresponding to 20 mg α-tocopherol equivalents; Cardi-E; Cardinova, Uppsala, Sweden) or 100 mg *all-rac*-α-tocopherol acetate corresponding to 45 mg α-tocopherol equivalents (E-vimin; Astra, Södertälje, Sweden).

All subjects gave informed consent, with permission to withdraw from the study at any time. The study was approved by the local ethics committee.

Blood collection and platelet preparation

Blood samples were collected from an antecubital vein before and after supplementation. The subjects were requested to fast for 12 h before blood sampling, and blood was drawn with the usual precautions required for the maintenance of platelet function. Ten milliliters of venous blood was collected into tubes, each containing 0.129 mol sodium citrate/L. The blood was separated by centrifugation at 200 × *g* for 10 min at 20 °C to obtain platelet-rich plasma. Platelet-rich plasma was further centrifuged at 1500 × *g* for 15 min at 20 °C, and the supernatant fluid was collected as platelet-poor plasma. The platelet count in the platelet-rich plasma was kept at ≈3×10^8 cells/mL.

Platelet aggregation

The method has been described earlier (12). In brief, platelets were stimulated by ADP (final concentration, 5 μmol/L) and phorbol 12-myristate 13-acetate (PMA; final concentration, 0.5 μmol/L). All aggregation studies were conducted in a 4-channel chronolog aggregometer (Bio/DATA Corp, Horsham, PA) in duplicate. After this procedure, the samples were centrifuged at 1500 × *g* for 15 min at 4°C. The supernatant fluid collected after ADP-induced aggregation was used to measure NO release. The platelet pellets after ADP stimulation were used for ecNOS or SOD Western blot analysis, and the platelet pellets after PMA stimulation were used for PKC Western blot analysis.

Determination of tocopherols in platelet-rich plasma

Amounts of α-, γ-, and δ-tocopherols in platelet-rich plasma were measured by HPLC with ultraviolet detection as described earlier (13, 14).

Determination of nitric oxide release

A colorimetric NO assay kit (15) was purchased from OXIS International, Inc (Portland, OR). This kit uses immunoaffinity-purified NADH-dependent Zea Mays nitrite reductase to determine total NO production after enzymatic conversion to nitrite. The procedures followed were the same as the manufacturer's description. Different concentrations (0, 10, 20, 50, 100, 200, 400, and 500 μmol/L) of nitrite were used as external standards. Nitrite was measured in the supernatant fluid of platelets at 540 nm in a microtiter reader and is expressed as nmol/3×10^8 platelets.

Platelet extraction

Platelets were harvested in 300 μL lysis buffer [20 mmol tris/L, pH 7.5; 1 mmol EDTA/L; 1% (by vol) Triton X-100; 150 mmol NaCl/L; 1 mmol PMSF/L; 1 μmol Pepstatin/L; 1% (by vol) Nonidet P-40; 10 μg leupeptin/mL; and 10 μg aprotinin/mL; all from Sigma-Aldrich Sweden AB, Stockholm]. After a 10-min incubation at 4 °C, platelets were disrupted by repeated aspiration through a 21-gauge needle and were centrifuged at 10 000 × *g* at 4 °C for 15 min. The supernatant fluid was used for Western blot analysis or immunoprecipitation.

Western blot for SOD, ecNOS, and PKC protein content

Western blot analysis was performed as described previously (16). Briefly, platelet lysates containing equal amounts of protein were resuspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and were boiled for 5 min. Samples were separated by SDS-PAGE (Cu/Zn SOD and PKC in a 12%-polyacrylamide gel and ecNOS in 7.5%-polyacrylamide gel) and were transferred to nitrocellulose membranes (Amersham Pharmacia Biotechnology, Uppsala, Sweden). After incubation in blocking solution (5% albumin; Sigma, St Louis), membranes were incubated with a primary antibody against Cu/Zn SOD (OXIS International Inc), ecNOS (Santa Cruz Biotechnology, Heidelberg, Germany), or PKC (isoforms α, β, γ; Santa Cruz Biotechnology) at dilutions of 1:500, 1:250, and 1:1000, respectively. Membranes were washed and incubated with horseradish peroxidase–conjugated secondary antibody (Amersham Pharmacia Biotechnology) to Cu/Zn SOD at 1:1000, ecNOS at 1:500, and PKC at 1:1000; the membranes were then detected by the enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotechnology). The intensity of the bands was analyzed with the use of an Apple Color One Scanner (Apple Computer, Inc, Cupertino, CA) and a Scion Image System (Scion Corporation, Frederick, MD).

Immunoprecipitation and Western blot for ecNOS and PKC activation in platelets

Activation of ecNOS and PKC in platelets was determined by measuring their phosphorylation. For identification of ecNOS and PKC phosphorylation, duplicated platelet lysates were subjected to immunoprecipitation and then to Western blot analysis, as described earlier (17). In brief, platelet lysates containing equal amounts of protein were immunoprecipitated with mouse monoclonal anti-PKC or rabbit polyclonal anti-ecNOS antibodies to human (from Santa Cruz Biotechnology, Santa Cruz, CA) for 3 h at 4 °C followed by absorption on Protein A/G Plus-Agarose (Santa Cruz Biotechnology) for 1 h at 4 °C. Precipitated samples were washed and recovered by centrifugation (1500 × *g*, 15 min, 4°C), after which the proteins were resuspended in SDS-PAGE sample buffer, boiled for 5 min, and assayed by Western blot analysis as described previously. Membranes were incubated with serine-threonine polyclonal antibodies at 1:500 and horseradish peroxidase–conjugated secondary antibodies at 1:500 (Amersham Pharmacia Biotechnology) and detected with the ECL system.

Statistical analysis

Data are presented as means ± SEMs. Statistical significance in multiple comparisons was determined by repeated-measures analysis of variance followed by post hoc Tukey's tests. P values < 0.05 were considered significant. The SPSS software package for WINDOWS 10.1 was used (SPSS Inc, Chicago).

RESULTS

Tocopherol and lipid concentrations

As shown in **Table 1**, after 8 wk of supplementation with mixed tocopherols, concentrations of α-, γ-, and δ-tocopherol in platelet-rich plasma were significantly increased. After

The American Journal of Clinical Nutrition

TABLE 1
Tocopherol concentrations in platelet-rich plasma and plasma lipid concentrations before and after 8 wk of supplementation with tocopherols[1]

	Mixed tocopherol group (n = 18)	α-Tocopherol group (n = 18)	Control group (n = 10)
α-Tocopherol (μmol/L)			
Baseline	13.24 ± 0.99	13.88 ± 1.13	13.54 ± 1.60
8 wk	18.13 ± 0.65[b,2]	21.23 ± 0.83[a,3]	16.46 ± 1.10[b]
γ-Tocopherol (μmol/L)			
Baseline	0.80 ± 0.10	1.05 ± 0.13	0.91 ± 0.13
8 wk	3.38 ± 0.32[a,3]	0.81 ± 0.07[b]	1.35 ± 0.22[b]
δ-Tocopherol (μmol/L)			
Baseline	0.00	0.00	0.00
8 wk	0.82 ± 0.20[a,3]	0.00[b]	0.17 ± 0.05[b]
Total cholesterol (mmol/L)			
Baseline	5.16 ± 0.15	5.20 ± 0.23	4.98 ± 0.27
8 wk	5.25 ± 0.17	4.91 ± 0.20	5.10 ± 0.31
Triacylglycerol (mmol/L)			
Baseline	0.90 ± 0.11	0.98 ± 0.06	1.00 ± 0.21
8 wk	1.08 ± 0.14	0.99 ± 0.11	0.99 ± 0.15

[1] $\bar{x}$ ± SEM. Means in the same row with different superscript letters are significantly different, $P < 0.05$.
[2,3] Significantly different from baseline (repeated-measures ANOVA followed by post hoc Tukey's test): [2] $P < 0.01$, [3] $P < 0.001$.

supplementation with α-tocopherol alone, α-tocopherol concentrations were increased; there were no significant changes in the control group. No significant changes were found in plasma total cholesterol or triacylglycerol concentrations in the 3 groups after supplementation.

Effect of tocopherols on platelet aggregation

There were no significant differences in platelet aggregation between the 3 groups before tocopherol supplementation. As shown in **Figure 1**, ADP-induced platelet aggregation decreased from 81.1 ± 3.4% to 69.8 ± 5.0% in the mixed tocopherol group ($P < 0.01$ for the change). No significant changes were found in the α-tocopherol group or in the control group. There were no significant changes in PMA-induced platelet aggregation in any of the groups.

Nitric oxide release from platelets and ecNOS activation in platelets

Supplementation with mixed tocopherols and α-tocopherol increased NO release from platelets. NO release was higher after supplementation in the mixed tocopherol group than in the group given α-tocopherol alone (**Figure 2**). ecNOS activation in platelets increased markedly in tocopherol-treated subjects (**Figure 3**). ecNOS phosphorylation showed a greater increase after supplementation with mixed tocopherols than after supplementation with α-tocopherol alone. No significant change in ecNOS protein content was observed in any of the groups.

Superoxide dismutase protein content in platelets

As shown in **Figure 4**, Cu/Zn SOD protein content was increased after tocopherol supplementation. There was no significant difference between the groups treated with mixed tocopherols or α-tocopherol alone.

Protein kinase C activation in platelets

PMA-stimulated PKC activation in platelets was reduced after the period of tocopherol supplementation. There was no

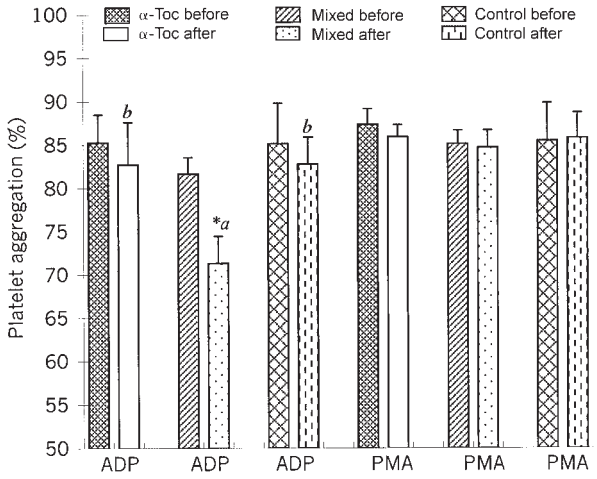

FIGURE 1. Mean (±SEM) ADP- and phorbol 12-myristate 13-acetate (PMA)-induced platelet aggregation before and after supplementation for 8 wk with α-tocopherol (α-Toc) or mixed tocopherols (Mixed). Mixed tocopherols but not α-tocopherol inhibited ADP-induced platelet aggregation. No significant change was found in PMA-induced platelet aggregation. n = 18 (α-tocopherol group), 18 (mixed group), and 10 (control group). For 8-wk data, means with different letters are significantly different, $P < 0.05$. *Significantly different from before supplementation, $P < 0.05$ (repeated-measures ANOVA followed by post hoc Tukey's test).

significant difference between the groups treated with mixed tocopherols or α-tocopherol alone. No significant change in PKC protein content was observed in any of the 3 groups, as shown in **Figure 5**.

DISCUSSION

The biological activity of tocopherols is expressed in international units (IU) or α-tocopherol equivalents (α-TE). One IU of vitamin E activity is defined as 1 mg *all-rac*-α-tocopherol

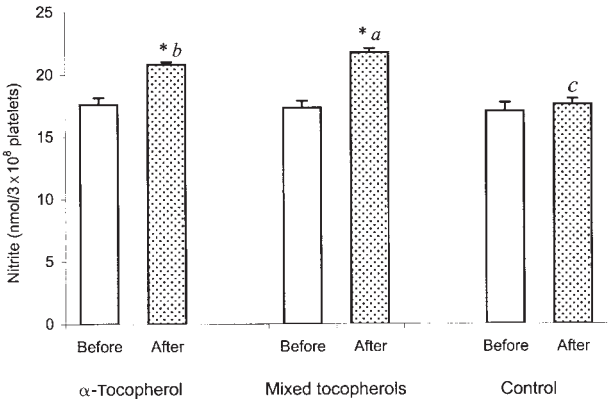

FIGURE 2. Mean (±SEM) nitric oxide (NO) release before and after supplementation for 8 wk with α-tocopherol or mixed tocopherols. Supplementation with mixed tocopherols and α-tocopherol increased NO release from platelets. Mixed tocopherols increased NO release more than did α-tocopherol. n = 18 (α-tocopherol group), 18 (mixed group), and 10 (control group). For 8-wk data, means with different letters are significantly different, $P < 0.05$. *Significantly different from before supplementation, $P < 0.01$ (repeated-measures ANOVA followed by post hoc Tukey's test).

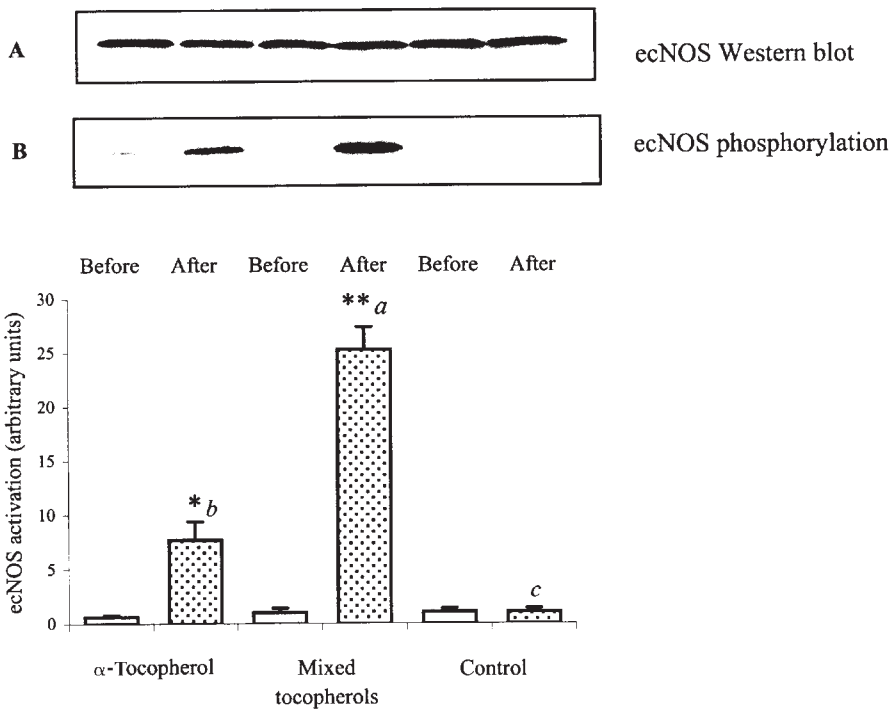

FIGURE 3. Mean (± SEM) endothelial constitutive nitric oxide synthase (ecNOS) protein content (A) and activation (phosphorylation; B) in platelets before and after supplementation for 8 wk with α-tocopherol or mixed tocopherols. There was no significant change in ecNOS protein content after tocopherol supplementation. Supplementation with tocopherols increased ecNOS activation. ecNOS activation was increased more by mixed tocopherols than by α-tocopherol. Data are based on 6 experiments. For 8-wk data, means with different letters are significantly different, $P < 0.01$. *,**Significantly different from before supplementation (repeated-measures ANOVA followed by post hoc Tukey's test): $^{*}P < 0.01$, $^{**}P < 0.001$.

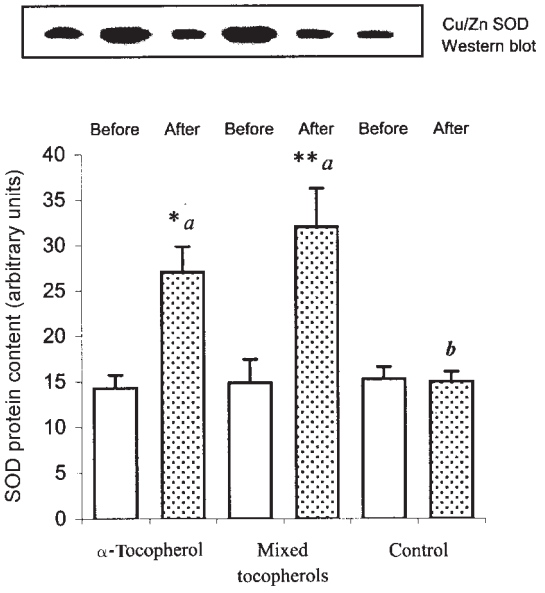

FIGURE 4. Mean (± SEM) Cu/Zn superoxide dismutase (SOD) protein content in platelets before and after supplementation for 8 wk with α-tocopherol or mixed tocopherols. Supplementation with mixed tocopherols and α-tocopherol increased Cu/Zn SOD protein content, but there was no significant difference between the groups. Data are based on 6 experiments. For 8-wk data, means with different letters are significantly different, $P < 0.01$. *,**Significantly different from before supplementation (repeated-measures ANOVA followed by post hoc Tukey's test): $^{*}P < 0.05$, $^{**}P < 0.01$.

acetate, or 0.67 mg *RRR*-α-tocopherol, according to official US Pharmacopoeia conversions. One milligram of the natural form *d*-α-tocopherol (*RRR*-α-tocopherol) is 1 mg α-TE and equal to 1.49 IU synthetic dl-α-tocopherol acetate (*all-rac*-α-tocopherol). *all-rac*-α-Tocopherol has one-half of the activity of the *RRR*-α-tocopherol found in foods or present with other 2*R*-stereoisomeric forms. On the basis of the new definition of vitamin E, 1 mg or IU *all-rac*-α-tocopherol contains the equivalent of 0.45 mg 2*R*-α-tocopherol (18). γ-Tocopherol showed just one-tenth of the activity of α-tocopherol in anti-sterility tests and the effect of δ-tocopherol was close to zero. α-TEs were defined as follows: α-tocopherol, mg × 1.0; γ-tocopherol, mg × 0.1; δ-tocopherol, mg × 0.03.

According to this official definition, even though the amount of tocopherols in milligrams was higher in the mixed tocopherol group in the present study, the number of IUs or α-TEs was less than one-half of that in the α-tocopherol group. The present results clearly show that the official formula for the biological activity of tocopherols cannot be used when determining the effects of tocopherols on platelet aggregation. We suppose that supplementation with the doses of mixed tocopherols (160 mg) and α-tocopherol (100 mg) used in this study was comparable, because it resulted in almost equal total tocopherol concentrations (α- + γ- + δ-tocopherol) in platelet-rich plasma of 22.33 and 22.01 μmol/L, respectively.

α-Tocopherol was found to inhibit platelet aggregation in some studies (19–22), whereas no effect was observed in other studies (23, 24). Previous in vitro studies by our research group showed that α-, γ-, and δ-tocopherol have similar effects on human

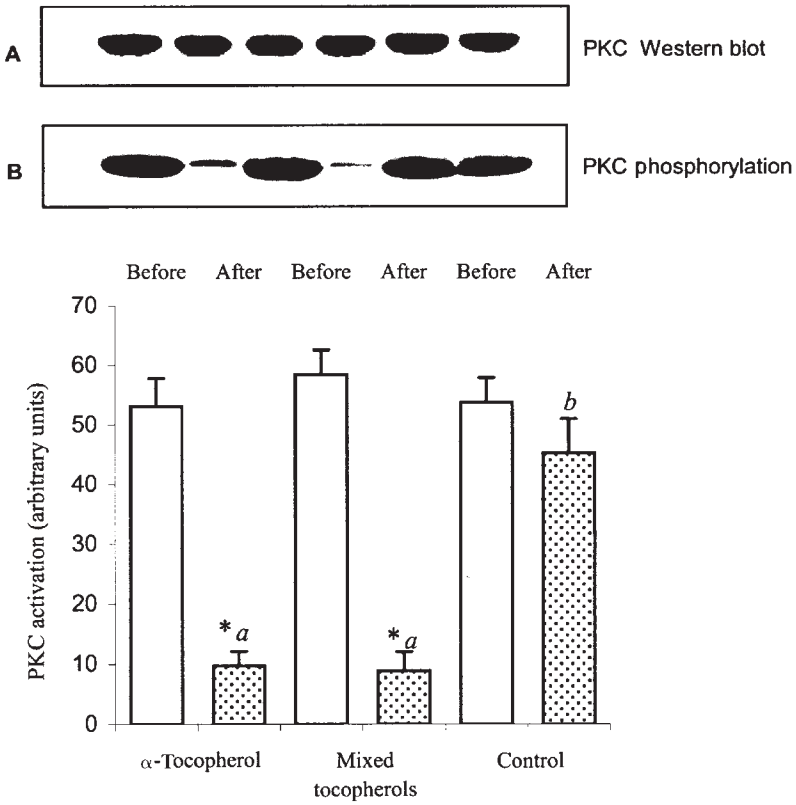

FIGURE 5. Mean (±SEM) protein kinase C (PKC) protein content (A) and activation (phosphorylation; B) in platelets before and after supplementation for 8 wk with α-tocopherol or mixed tocopherols. There was no significant change in PKC protein content after tocopherol supplementation. Supplementation with mixed tocopherols and α-tocopherol decreased PKC activation, but there was no significant difference between the groups. Data are based on 6 experiments. For 8-wk data, means with different letters are significantly different, $P < 0.01$. *Significantly different from before supplementation, $P < 0.01$ (repeated-measures ANOVA followed by post hoc Tukey's test).

platelet aggregation and that a combination of the different tocopherols has a synergistic platelet inhibitory effect. This synergistic effect may explain the better effect of mixed tocopherols than of α-tocopherol alone in the present study. We also showed that the cellular uptake of mixed tocopherols is much higher than that of α-tocopherol after incubation of the 2 preparations at the same molar concentration (12, 17). In most studies of inhibition of platelet aggregation by α-tocopherol, nonphysiologic concentrations or high doses were used. In the present study, a relatively low dose of α-tocopherol was used. This may explain why α-tocopherol alone had no effect on platelet aggregation in our subjects.

The mechanism by which tocopherols inhibit platelet aggregation is not completely known. One obvious mode of action is related to NO bioactivity. Decreased bioavailability of NO is a characteristic feature in patients with coronary artery disease, and impaired platelet NO production predicts acute coronary syndromes (25). Platelet-derived NO has been found to inhibit platelet aggregation and reduce platelet recruitment to a growing thrombus (26). Incorporation of tocopherol modulates the balance between NO and superoxide in human platelets (27). α-Tocopherol might increase platelet NO release by its free radical scavenging activity and by preventing its quenching by peroxyl radicals (28, 29). NO is formed by nitric oxide synthase, a process in which ecNOS plays a crucial role. In mice lacking the gene encoding ecNOS, platelets lack stimulation-induced NO release (30). It was recently confirmed in several studies that phosphorylation of Ser1177 in human ecNOS (Ser1179 in bovine ecNOS) leads to ecNOS activation and enhances the ability of the enzyme to generate NO in endothelial cells. One compensatory mechanism is that γ-tocopherol can be nitrated and thus may react with NO, possibly depleting NO and causing an up-regulation of NO synthesis (31). Previous studies by our research group showed that a γ-tocopherol–rich preparation increased ecNOS activity and NO generation in rat aorta (17, 32). Also, α-, γ-, and δ-tocopherol increased NO release and ecNOS activity, and a combination of the 3 isoforms had a synergistic effect. Thus, we suggest that increased NO release due to increased ecNOS activity in platelets may be a major mechanism underlying the effect of the mixed tocopherol preparation on platelet aggregation.

Oxidative stress and antioxidant status are important in platelet function. In patients with coronary artery disease, decreased plasma and platelet antioxidant activity is associated with increased platelet aggregability (33). Lipid peroxidation (34, 35) and superoxide production (36, 37) also augment platelet aggregation. Loss of NO bioactivity is attributable to increased oxidative stress, particularly to increased production of superoxide anions and the accumulation of products of lipid peroxidation. Previous studies showed that SOD inhibits platelet activation (38) and that inhibition of platelet aggregation after tocopherol intake is greater in subjects with low antioxidant status (39). In our study, the tocopherols increased the SOD protein amount. These findings suggest that tocopherol supplementation up-regulates

The American Journal of Clinical Nutrition

intrinsic SOD expression at the protein level, a process that may be an important mechanism underlying the effect of tocopherols on platelet aggregation.

In a previous study (40), it was reported that α-tocopherol inactivates cellular PKC-α by changing its phosphorylation state. Freedman et al (21, 41) suggested that α-tocopherol inhibits platelet aggregation by a PKC-dependent mechanism and in a dose-dependent manner, which stemmed from the nonantioxidant actions of tocopherol. Stimulation of platelets by the agonist PMA induced platelet protein phosphorylation of PKC, and in α-tocopherol–loaded platelets the phosphorylation was reduced. Ohmori et al (42) showed that PMA-induced platelet aggregation was weak despite marked PKC activation. Furthermore, PKC was shown to be little involved in ADP-induced primary aggregation of human platelets (43). In the present study, PKC phosphorylation was equally decreased in both tocopherol-treated groups. The tocopherols did not inhibit PKC expression but inhibited PKC activation at a cellular level by causing dephosphorylation. Although intake of α-tocopherol resulted in a marked decrease in PKC activation, it had no effect on platelet aggregation, arguing against a major role of PKC in platelet aggregation. In our study, PMA-induced platelet aggregation was not affected by any of the tocopherols despite a decrease in phosphorylation after tocopherol supplementation. A possible explanation might be that higher doses of tocopherols are needed to overcome the effect of PMA. Further research is needed to clarify this.

In our study, mixed tocopherols inhibited ADP-induced platelet aggregation but not PMA-induced aggregation. Thus, we suggest that mixed tocopherols may inhibit platelet aggregation by an ADP-related mechanism.

In conclusion, mixed tocopherols but not α-tocopherol prevented ADP-induced platelet aggregation. The effects of mixed tocopherols and of α-tocopherol were associated with increases in NO release, ecNOS activation, and SOD protein content and with a decrease in PKC activation in platelets. Mixed tocopherols were more potent than α-tocopherol alone in modulating NO release and ecNOS activation, which may contribute to the effect of mixed tocopherols on platelet aggregation.

ML contributed to data collection, data analysis, and manuscript writing; ML, AW, and CO-M performed laboratory assays; RW prepared reagents and contributed to sample collection; and TS initiated the study, contributed to manuscript writing, and supervised the project.

REFERENCES

1. Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC. Vitamin E consumption and the risk of coronary heart disease in men. N Engl J Med 1993;328:1450–6.
2. Stampfer MJ, Hennekens CH, Manson JE, Colditz GA, Rosner B, Willett WC. Vitamin E consumption and the risk of coronary disease in women. N Engl J Med 1993;328:1444–9.
3. Kushi LH, Folsom AR, Prineas RJ, Mink PJ, Wu Y, Bostick RM. Dietary antioxidant vitamins and death from coronary heart disease in postmenopausal women. N Engl J Med 1996;334:1156–62.
4. Losonczy KG, Harris TB, Havlik RJ. Vitamin E and vitamin C supplement use and risk of all-cause and coronary heart disease mortality in older persons: the Established Populations for Epidemiologic Studies of the Elderly. Am J Clin Nutr 1996;64:190–6.
5. Yusuf S, Dagenais G, Pogue J, Bosch J, Sleight P. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. N Engl J Med 2000;342:154–60.
6. GISSI. Dietary supplementation with n−3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. Lancet 1999;354:447–55.
7. Wolf G. γ-Tocopherol: an efficient protector of lipids against nitric oxide-initiated peroxidative damage. Nutr Rev 1997;55:376–8.
8. Ohrvall M, Sundlof G, Vessby B. Gamma, but not alpha, tocopherol levels in serum are reduced in coronary heart disease patients. J Intern Med 1996;239:111–7.
9. Conti CR, Mehta JL. Acute myocardial ischemia: role of atherosclerosis, thrombosis, platelet activation, coronary vasospasm, and altered arachidonic acid metabolism. Circulation 1987;75:V84–95.
10. Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes. N Engl J Med 1992;326:242–50.
11. Handin RI. Platelets and coronary artery disease. N Engl J Med 1996;334:1126–7.
12. Saldeen T, Li D, Mehta JL. Differential effects of alpha- and gamma-tocopherol on low-density lipoprotein oxidation, superoxide activity, platelet aggregation and arterial thrombogenesis. J Am Coll Cardiol 1999;34:1208–15.
13. Ohrvall M, Tengblad S, Vessby B. Lower tocopherol serum levels in subjects with abdominal adiposity. J Intern Med 1993;234:53–60.
14. American Oil Chemist's Society. Determination of tocopherols and tocotrienols in vegetable oils and fats by HPLC. Sampling and analysis of commercial fats and oils. Champaign, IL: American Oil Chemist's Society, 1990. (Official method Ce 8–89, revised.)
15. Schmidt HHW. Determination of nitric oxide via measurement of nitrite and nitrate in culture media. Biochemica 1995;2:22–3.
16. Li D, Tomson K, Yang B, Mehta P, Croker BP, Mehta JL. Modulation of constitutive nitric oxide synthase, bcl-2 and Fas expression in cultured human coronary endothelial cells exposed to anoxia-reoxygenation and angiotensin II: role of AT1 receptor activation. Cardiovasc Res 1999;41:109–15.
17. Li D, Saldeen T, Romeo F, Mehta JL. Different isoforms of tocopherols enhance nitric oxide synthase phosphorylation and inhibit human platelet aggregation and lipid peroxidation: implications in therapy with vitamin E. J Cardiovasc Pharmacol Ther 2001;6:155–61.
18. Institute of Medicine, Food and Nutrition Board. Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids.: Washington, DC: National Academy Press, 2000.
19. Steiner M, Anastasi J. Vitamin E. An inhibitor of the platelet release reaction. J Clin Invest 1976;57:732–7.
20. Srivastava KC. Vitamin E exerts antiaggregatory effects without inhibiting the enzymes of the arachidonic acid cascade in platelets. Prostaglandins Leukot Med 1986;21:177–85.
21. Freedman JE, Farhat JH, Loscalzo J, Keaney JF Jr. Alpha-tocopherol inhibits aggregation of human platelets by a protein kinase C-dependent mechanism. Circulation 1996;94:2434–40.
22. Mabile L, Bruckdorfer KR, Rice-Evans C. Moderate supplementation with natural alpha-tocopherol decreases platelet aggregation and low-density lipoprotein oxidation. Atherosclerosis 1999;147:177–85.
23. Stampfer MJ, Jakubowski JA, Faigel D, Vaillancourt R, Deykin D. Vitamin E supplementation effect on human platelet function, arachidonic acid metabolism, and plasma prostacyclin levels. Am J Clin Nutr 1988;47:700–6.
24. Kockmann V, Vericel E, Croset M, Lagarde M. Vitamin E fails to alter the aggregation and the oxygenated metabolism of arachidonic acid in normal human platelets. Prostaglandins 1988;36:607–20.
25. Freedman JE, Ting B, Hankin B, Loscalzo J, Keaney JF Jr, Vita JA. Impaired platelet production of nitric oxide predicts presence of acute coronary syndromes. Circulation 1998;98:1481–6.
26. Freedman JE, Loscalzo J, Barnard MR, Alpert C, Keaney JF, Michelson AD.

Nitric oxide released from activated platelets inhibits platelet recruitment. J Clin Invest 1997;100:350–6.
27. Freedman JE, Li L, Sauter R, Keaney JJ. α-Tocopherol and protein kinase C inhibition enhance platelet-derived nitric oxide release. FASEB J 2000;14:2377–9.
28. Huie RE, Padmaja S. The reaction of NO with superoxide. Free Radic Res Commun 1993;18:195–9.
29. Rubbo H, Radi R, Trujillo M, et al. Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. J Biol Chem 1994;269:26066–75.
30. Freedman JE, Sauter R, Battinelli EM, et al. Deficient platelet-derived nitric oxide and enhanced hemostasis in mice lacking the NOS III gene. Circ Res 1999;84:1416–21.
31. Christen S, Woodall AA, Shigenaga MK, Southwell-Keely PT, Duncan MW, Ames BN. Gamma-tocopherol traps mutagenic electrophiles such as NO(X) and complements alpha-tocopherol: physiological implications. Proc Natl Acad Sci U S A 1997;94: 3217–22.
32. Li D, Saldeen T, Romeo F, Mehta JL. Relative effects of alpha- and gamma-tocopherol on low-density lipoprotein oxidation and superoxide dismutase and nitric oxide synthase activity and protein expression in rats. J Cardiovasc Pharmacol Ther 1999;4:219–26.
33. Buczynski A, Wachowicz B, Kedziora-Kornatowska K, Tkaczewski W, Kedziora J. Changes in antioxidant enzymes activities, aggregability and malonyldialdehyde concentration in blood platelets from patients with coronary heart disease. Atherosclerosis 1993;100: 223–8.
34. Okuma M, Steiner M, Baldini G. Studies on lipid peroxides in platelets. II. Effect of aggregating agents and platelet antibody. J Lab Clin Med 1971;77:728–42.
35. Muller M, Sorrell TC. Oxidative stress and the mobilisation of arachidonic acid in stimulated human platelets: role of hydroxyl radical. Prostaglandins 1997;54:493–509.
36. Handin RI, Karabin R, Boxer GJ. Enhancement of platelet function by superoxide anion. J Clin Invest 1977;59:959–65.
37. Freedman J, Keaney J. NO and superoxide in human platelets. In: Packer L, ed. Nitric oxide, part C. Biological and antioxidant activities. San Diego: Academic Press, 1999:61–7.
38. Mehta J, Li D, Mehta JL. Vitamins C and E prolong time to arterial thrombosis in rats. J Nutr 1999;129:109–12.
39. Salonen JT, Salonen R, Seppanen K, et al. Effects of antioxidant supplementation on platelet function: a randomized pair-matched, placebo-controlled, double-blind trial in men with low antioxidant status. Am J Clin Nutr 1991;53:1222–9.
40. Ricciarelli R, Tasinato A, Clement S, Ozer NK, Boscoboinik D, Azzi A. α-Tocopherol specifically inactivates cellular protein kinase C alpha by changing its phosphorylation state. Biochem J 1998; 334:243–9.
41. Freedman JE, Keaney JF Jr. Vitamin E inhibition of platelet aggregation is independent of antioxidant activity. J Nutr 2001;131: 374S–7S.
42. Ohmori T, Yatomi Y, Asazuma N, Satoh K, Ozaki Y. Involvement of proline-rich tyrosine kinase 2 in platelet activation: tyrosine phosphorylation mostly dependent on alphaIIbbeta3 integrin and protein kinase C, translocation to the cytoskeleton and association with Shc through Grb2. Biochem J 2000;347:561–9.
43. Packham MA, Livne AA, Ruben DH, Rand ML. Activation of phospholipase C and protein kinase C has little involvement in ADP-induced primary aggregation of human platelets: effects of diacylglycerols, the diacylglycerols, the diacylglycerol kinase inhibitor R59022, staurosporine and okadaic acid. Biochem J 1993;290: 849–56.