

# Mixed tocopherols inhibit platelet aggregation in humans: potential mechanisms<sup>1-3</sup>

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  $\alpha$ -tocopherol on cardiovascular events. Absence of tocopherols other than  $\alpha$ -tocopherol in the clinical studies may account for the conflicting results.

**Objective:** This study compared the effect of a mixed tocopherol preparation rich in  $\gamma$ -tocopherol with that of  $\alpha$ -tocopherol on platelet aggregation in humans and addressed the potential mechanisms of the effect.

**Design:** Forty-six subjects were randomly divided into 3 groups:  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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,  $\alpha$ -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  $\alpha$ -tocopherol on cardiovascular events and cardiac death. In the clinical studies, the vitamin E preparation contained  $\alpha$ -tocopherol alone, whereas

vitamin E in food consists of several different tocopherols. Thus, absence of tocopherols other than  $\alpha$ -tocopherol in the preparations used in the clinical studies may account for the conflicting results. Among the other tocopherols,  $\gamma$ -tocopherol in particular has been shown to have potent antioxidant effects (7), and  $\gamma$ -tocopherol, but not  $\alpha$ -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  $\gamma$ -tocopherol was more potent than  $\alpha$ -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  $\gamma$ -tocopherol-rich preparation of mixed tocopherols with that of  $\alpha$ -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  $\pm$  1.5 y) were randomly assigned to 3 groups: 18 subjects were given  $\alpha$ -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

<sup>1</sup> From the Department of Surgical Sciences, Section of Forensic Medicine, University of Uppsala, Sweden.

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<sup>3</sup> 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.

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(100 mg  $\gamma$ -, 40 mg  $\delta$ -, and 20 mg  $\alpha$ -tocopherol corresponding to 20 mg  $\alpha$ -tocopherol equivalents; Cardi-E; Cardinova, Uppsala, Sweden) or 100 mg *all-rac*- $\alpha$ -tocopherol acetate corresponding to 45 mg  $\alpha$ -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  $200 \times g$  for 10 min at  $20^\circ\text{C}$  to obtain platelet-rich plasma. Platelet-rich plasma was further centrifuged at  $1500 \times g$  for 15 min at  $20^\circ\text{C}$ , and the supernatant fluid was collected as platelet-poor plasma. The platelet count in the platelet-rich plasma was kept at  $\approx 3 \times 10^8$  cells/mL.

### Platelet aggregation

The method has been described earlier (12). In brief, platelets were stimulated by ADP (final concentration,  $5 \mu\text{mol/L}$ ) and phorbol 12-myristate 13-acetate (PMA; final concentration,  $0.5 \mu\text{mol/L}$ ). 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  $1500 \times g$  for 15 min at  $4^\circ\text{C}$ . 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  $\alpha$ -,  $\gamma$ -, and  $\delta$ -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  $500 \mu\text{mol/L}$ ) 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  $\text{nmol}/3 \times 10^8$  platelets.

### Platelet extraction

Platelets were harvested in  $300 \mu\text{L}$  lysis buffer [ $20 \text{ mmol tris/L}$ , pH 7.5;  $1 \text{ mmol EDTA/L}$ ; 1% (by vol) Triton X-100;  $150 \text{ mmol NaCl/L}$ ;  $1 \text{ mmol PMSF/L}$ ;  $1 \mu\text{mol Pepstatin/L}$ ; 1% (by vol) Nonidet P-40;  $10 \mu\text{g leupeptin/mL}$ ; and  $10 \mu\text{g aprotinin/mL}$ ; all from Sigma-Aldrich Sweden AB, Stockholm]. After a 10-min incubation at  $4^\circ\text{C}$ , platelets were disrupted by repeated aspiration through a 21-gauge needle and were centrifuged at  $10000 \times g$  at  $4^\circ\text{C}$  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  $\alpha$ ,  $\beta$ ,  $\gamma$ ; 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  $4^\circ\text{C}$  followed by absorption on Protein A/G Plus-Agarose (Santa Cruz Biotechnology) for 1 h at  $4^\circ\text{C}$ . Precipitated samples were washed and recovered by centrifugation ( $1500 \times g$ , 15 min,  $4^\circ\text{C}$ ), 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  $\pm$  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  $\alpha$ -,  $\gamma$ -, and  $\delta$ -tocopherol in platelet-rich plasma were significantly increased. After

**TABLE 1**

Tocopherol concentrations in platelet-rich plasma and plasma lipid concentrations before and after 8 wk of supplementation with tocopherols<sup>1</sup>

	Mixed tocopherol group (n = 18)	$\alpha$ -Tocopherol group (n = 18)	Control group (n = 10)
$\alpha$ -Tocopherol ( $\mu\text{mol/L}$ )			
Baseline	13.24 $\pm$ 0.99	13.88 $\pm$ 1.13	13.54 $\pm$ 1.60
8 wk	18.13 $\pm$ 0.65 <sup>b,2</sup>	21.23 $\pm$ 0.83 <sup>a,3</sup>	16.46 $\pm$ 1.10 <sup>b</sup>
$\gamma$ -Tocopherol ( $\mu\text{mol/L}$ )			
Baseline	0.80 $\pm$ 0.10	1.05 $\pm$ 0.13	0.91 $\pm$ 0.13
8 wk	3.38 $\pm$ 0.32 <sup>a,3</sup>	0.81 $\pm$ 0.07 <sup>b</sup>	1.35 $\pm$ 0.22 <sup>b</sup>
$\delta$ -Tocopherol ( $\mu\text{mol/L}$ )			
Baseline	0.00	0.00	0.00
8 wk	0.82 $\pm$ 0.20 <sup>a,3</sup>	0.00 <sup>b</sup>	0.17 $\pm$ 0.05 <sup>b</sup>
Total cholesterol (mmol/L)			
Baseline	5.16 $\pm$ 0.15	5.20 $\pm$ 0.23	4.98 $\pm$ 0.27
8 wk	5.25 $\pm$ 0.17	4.91 $\pm$ 0.20	5.10 $\pm$ 0.31
Triacylglycerol (mmol/L)			
Baseline	0.90 $\pm$ 0.11	0.98 $\pm$ 0.06	1.00 $\pm$ 0.21
8 wk	1.08 $\pm$ 0.14	0.99 $\pm$ 0.11	0.99 $\pm$ 0.15

<sup>1</sup> $\bar{x} \pm \text{SEM}$ . Means in the same row with different superscript letters are significantly different,  $P < 0.05$ .

<sup>2,3</sup>Significantly different from baseline (repeated-measures ANOVA followed by post hoc Tukey's test): <sup>2</sup> $P < 0.01$ , <sup>3</sup> $P < 0.001$ .

supplementation with  $\alpha$ -tocopherol alone,  $\alpha$ -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  $\pm$  3.4% to 69.8  $\pm$  5.0% in the mixed tocopherol group ( $P < 0.01$  for the change). No significant changes were found in the  $\alpha$ -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 eNOS activation in platelets

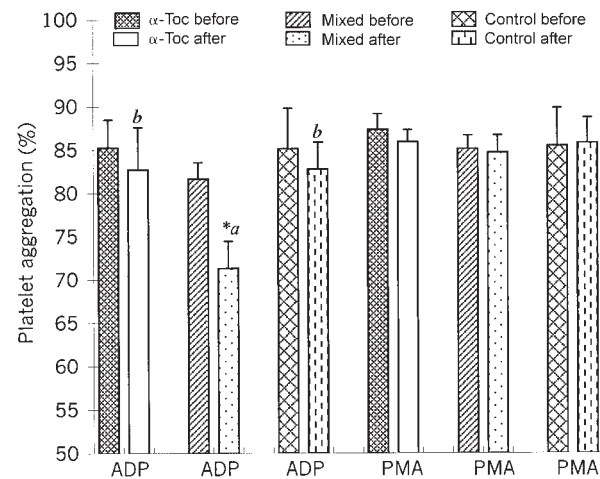
Supplementation with mixed tocopherols and  $\alpha$ -tocopherol increased NO release from platelets. NO release was higher after supplementation in the mixed tocopherol group than in the group given  $\alpha$ -tocopherol alone (**Figure 2**). eNOS activation in platelets increased markedly in tocopherol-treated subjects (**Figure 3**). eNOS phosphorylation showed a greater increase after supplementation with mixed tocopherols than after supplementation with  $\alpha$ -tocopherol alone. No significant change in eNOS 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  $\alpha$ -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

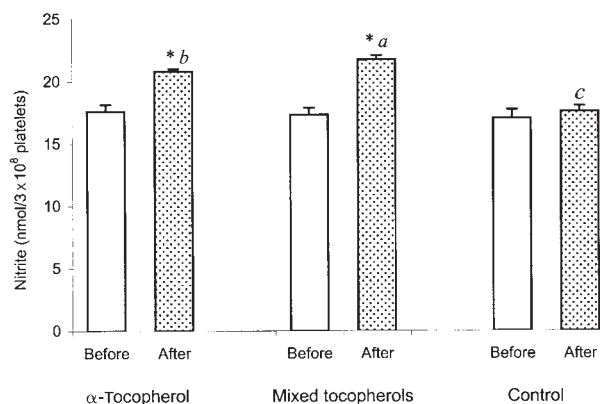


**FIGURE 1.** Mean ( $\pm$ SEM) ADP- and phorbol 12-myristate 13-acetate (PMA)-induced platelet aggregation before and after supplementation for 8 wk with  $\alpha$ -tocopherol ( $\alpha$ -Toc) or mixed tocopherols (Mixed). Mixed tocopherols but not  $\alpha$ -tocopherol inhibited ADP-induced platelet aggregation. No significant change was found in PMA-induced platelet aggregation.  $n = 18$  ( $\alpha$ -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).

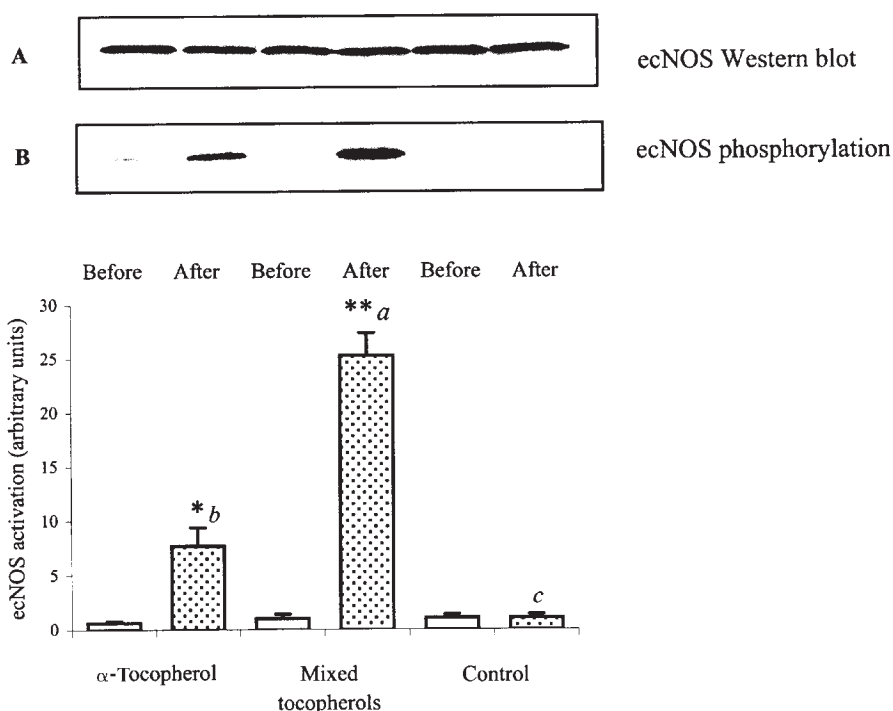
significant difference between the groups treated with mixed tocopherols or  $\alpha$ -tocopherol alone. No significant change in PKC protein content was observed in any of the 3 groups, as shown in **Figure 5**.

#### DISCUSSION

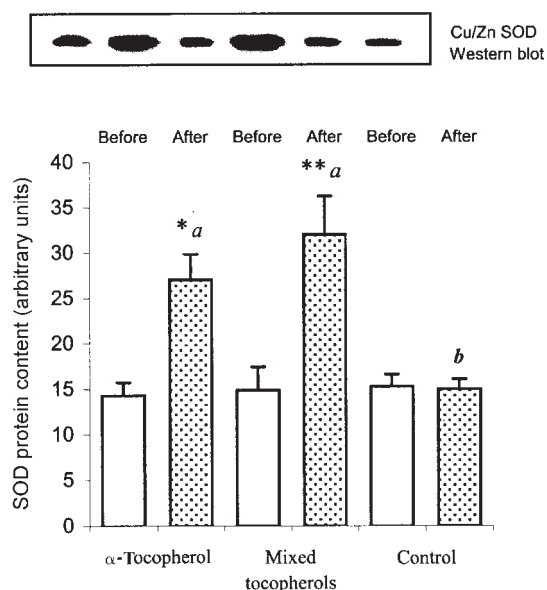
The biological activity of tocopherols is expressed in international units (IU) or  $\alpha$ -tocopherol equivalents ( $\alpha$ -TE). One IU of vitamin E activity is defined as 1 mg *all-rac*- $\alpha$ -tocopherol



**FIGURE 2.** Mean ( $\pm$ SEM) nitric oxide (NO) release before and after supplementation for 8 wk with  $\alpha$ -tocopherol or mixed tocopherols. Supplementation with mixed tocopherols and  $\alpha$ -tocopherol increased NO release from platelets. Mixed tocopherols increased NO release more than did  $\alpha$ -tocopherol.  $n = 18$  ( $\alpha$ -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 ( $\pm$  SEM) endothelial constitutive nitric oxide synthase (ecNOS) protein content (A) and activation (phosphorylation; B) in platelets before and after supplementation for 8 wk with  $\alpha$ -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  $\alpha$ -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): \* $P < 0.01$ , \*\* $P < 0.001$ .

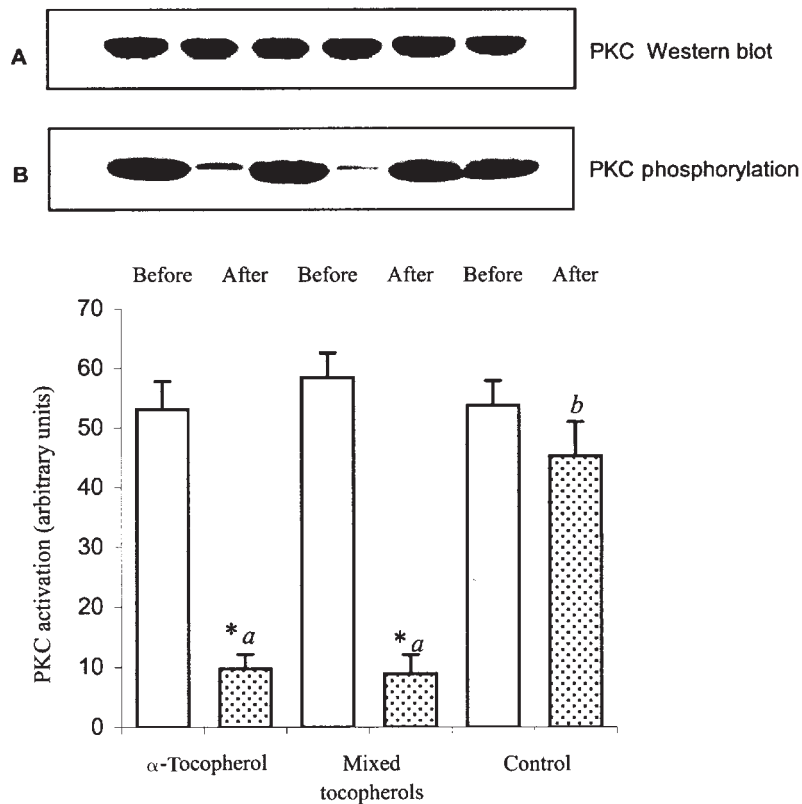


**FIGURE 4.** Mean ( $\pm$  SEM) Cu/Zn superoxide dismutase (SOD) protein content in platelets before and after supplementation for 8 wk with  $\alpha$ -tocopherol or mixed tocopherols. Supplementation with mixed tocopherols and  $\alpha$ -tocopherol increased Cu/Zn SOD protein content, but there was 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*- $\alpha$ -tocopherol, according to official US Pharmacopoeia conversions. One milligram of the natural form *d*- $\alpha$ -tocopherol (*RRR*- $\alpha$ -tocopherol) is 1 mg  $\alpha$ -TE and equal to 1.49 IU synthetic *dl*- $\alpha$ -tocopherol acetate (*all-rac*- $\alpha$ -tocopherol). *all-rac*- $\alpha$ -Tocopherol has one-half of the activity of the *RRR*- $\alpha$ -tocopherol found in foods or present with other *2R*-stereoisomeric forms. On the basis of the new definition of vitamin E, 1 mg or IU *all-rac*- $\alpha$ -tocopherol contains the equivalent of 0.45 mg *2R*- $\alpha$ -tocopherol (18).  $\gamma$ -Tocopherol showed just one-tenth of the activity of  $\alpha$ -tocopherol in anti-sterility tests and the effect of  $\delta$ -tocopherol was close to zero.  $\alpha$ -TEs were defined as follows:  $\alpha$ -tocopherol, mg  $\times$  1.0;  $\gamma$ -tocopherol, mg  $\times$  0.1;  $\delta$ -tocopherol, mg  $\times$  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  $\alpha$ -TEs was less than one-half of that in the  $\alpha$ -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  $\alpha$ -tocopherol (100 mg) used in this study was comparable, because it resulted in almost equal total tocopherol concentrations ( $\alpha$ - +  $\gamma$ - +  $\delta$ -tocopherol) in platelet-rich plasma of 22.33 and 22.01  $\mu$ mol/L, respectively.

$\alpha$ -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  $\alpha$ -,  $\gamma$ -, and  $\delta$ -tocopherol have similar effects on human



**FIGURE 5.** Mean ( $\pm$  SEM) protein kinase C (PKC) protein content (A) and activation (phosphorylation; B) in platelets before and after supplementation for 8 wk with  $\alpha$ -tocopherol or mixed tocopherols. There was no significant change in PKC protein content after tocopherol supplementation. Supplementation with mixed tocopherols and  $\alpha$ -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  $\alpha$ -tocopherol alone in the present study. We also showed that the cellular uptake of mixed tocopherols is much higher than that of  $\alpha$ -tocopherol after incubation of the 2 preparations at the same molar concentration (12, 17). In most studies of inhibition of platelet aggregation by  $\alpha$ -tocopherol, nonphysiologic concentrations or high doses were used. In the present study, a relatively low dose of  $\alpha$ -tocopherol was used. This may explain why  $\alpha$ -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).  $\alpha$ -Tocopherol might increase platelet NO release by its free radical scavenging activity and by preventing its quenching by peroxy radicals (28, 29). NO is formed by nitric oxide synthase, a process in which eNOS plays a crucial role. In mice lacking the gene encoding eNOS, platelets lack stimulation-induced NO release (30). It was recently confirmed in several studies that phosphorylation of Ser1177 in


human eNOS (Ser1179 in bovine eNOS) leads to eNOS activation and enhances the ability of the enzyme to generate NO in endothelial cells. One compensatory mechanism is that  $\gamma$ -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  $\gamma$ -tocopherol-rich preparation increased eNOS activity and NO generation in rat aorta (17, 32). Also,  $\alpha$ -,  $\gamma$ -, and  $\delta$ -tocopherol increased NO release and eNOS activity, and a combination of the 3 isoforms had a synergistic effect. Thus, we suggest that increased NO release due to increased eNOS 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

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  $\alpha$ -tocopherol inactivates cellular PKC- $\alpha$  by changing its phosphorylation state. Freedman et al (21, 41) suggested that  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -tocopherol prevented ADP-induced platelet aggregation. The effects of mixed tocopherols and of  $\alpha$ -tocopherol were associated with increases in NO release, eNOS activation, and SOD protein content and with a decrease in PKC activation in platelets. Mixed tocopherols were more potent than  $\alpha$ -tocopherol alone in modulating NO release and eNOS 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.

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