Vitamin E Inhibition of Platelet Aggregation Is Independent of Antioxidant Activity$^{1,2}$

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ABSTRACT Vitamin E is the principal lipid-soluble antioxidant in human plasma, and some studies indicate that it may provide cardiovascular protection. To investigate putative mechanisms for vitamin E in this regard, the effect of vitamin E on vascular function and platelet aggregation was examined. In animal models of endothelial dysfunction, vitamin E improved the activity of endothelium-derived nitric oxide, and this effect was not dependent upon the antioxidant protection of LDL. In fact, vitamin E improved endothelial function in part due to the inhibition of protein kinase C (PKC) stimulation. This activity of vitamin E was examined in platelets, and vitamin E inhibited platelet aggregation in part through a mechanism that involves PKC. Moreover, the platelet inhibitory activity of vitamin E was independent of its antioxidant action because platelet inhibition was still observed with isoforms of vitamin E that were devoid of antioxidant activity. J. Nutr. 131: 374S–377S, 2001.

KEY WORDS: vitamins • antioxidants • platelets • platelet aggregation inhibitors • thrombosis

Vitamin E is the principal lipid-soluble antioxidant in human plasma and lipoproteins. Epidemiologic studies have shown that dietary vitamin E consumption is inversely associated with the development of coronary artery disease in both men and women [reviewed in Stocker (1999)]. Intervention trials, however, have been mixed; some have demonstrated a protective effect of vitamin E (Stephens et al. 1996), whereas others have not ( Yusuf et al. 2000). One proposed mechanism for the putative cardioprotective effect of vitamin E has been the inhibition of LDL oxidation ( Stephens et al. 1996). However, the age of patients participating in intervention trials dictates the presence of established atherosclerosis ( Stary 1983); therefore, any putative action of vitamin E must involve events beyond the protection of LDL oxidation.

One important component of cardiovascular disease is the concept of atherosclerotic lesion “activation” ( Libby 1995). According to this concept, atherosclerotic lesions precipitate clinical events (i.e., become “active”) only when the normal homeostatic mechanisms of the arterial wall can no longer compensate for the atherosclerotic disease process. One important component of vascular homeostasis that is known to be abnormal in atherosclerosis is the bioactivity of endothelium-derived nitric oxide ( NO) ( Diaz et al. 1997). In most animal and some human studies, vitamin E has been shown to normalize the bioactivity of NO [reviewed in Duffy et al. (1999)]. The precise mechanism for these observations is not completely known, but one component appears to involve the activity of vitamin E in inhibiting protein kinase C ( PKC)4 stimulation (Keaney et al. 1996).

We also examined the activity of vitamin E with respect to platelet aggregation, another important aspect of vascular homeostasis. We found that platelet incorporation of vitamin E both in vitro and in vivo leads to dose-dependent inhibition of platelet aggregation in response to agonists such as arachidonic acid and phorbol ester (Freedman et al. 1996). Moreover, this effect of vitamin E in inhibiting platelet aggregation appears to be PKC dependent ( Freedman et al. 1996). We report here further investigations of platelet aggregation and vitamin E, which clearly demonstrate that the antioxidant activity of vitamin E is not a requirement for inhibition of aggregation.

MATERIALS AND METHODS

Materials. HEPES buffer solution (HBS), pH 7.35, consisted of 5.8 mmol/L sodium HEPES, 140 mmol/L NaCl, 6.1 mmol/L KCl, 2.5 mmol/L MgSO4, 2.4 mmol/L Na2SO4, 59 μmol/L bovine serum albumin, 0.1 mmol/L CaCl2, 0.5 mmol/L EGTA, 0.5 mmol/L MgCl2, 0.3 mmol/L 3isocitric acid, 0.3 mmol/L 6-isocitric acid, 0.1 mmol/L 3-phosphoglycerate, 0.3 mmol/L 6-phosphoglycerate, 0.1 mol/L 3PGA, 0.1 mmol/L glucose, 0.1 mmol/L ATP, 0.8 mmol/L phosphocreatine, 0.1 mmol/L creatine kinase (EC 3.1.3.8), 0.5 mmol/L [2,6-dichlorophenolindophenol], and 0.1 mmol/L inosine monophosphate.

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albumin and 5.6 mmol/L dextrose. RRR-α-Tocopherol (natural stereoisomer), its acetate and quinone forms were gifts from Henkel (Kansas, MO). All other compounds were purchased from Sigma (St. Louis, MO). Tocopherol isoforms were dissolved in 95% ethanol, and the concentrations of the tocopherol compounds were confirmed by spectrophotometric measurement using the corresponding extinction coefficient.

Preparation of platelet-rich plasma, gel-filtered platelets and platelet aggregation. Venous blood was obtained from volunteers who were not consuming vitamin supplements or aspirin as described (Freedman et al. 1996). Platelet-rich plasma (PRP) and gel-filtered platelets (GFP) were prepared from venous blood by centrifugation and Sepharose 2B chromatography as described (Freedman et al. 1996). For aggregations after gel-filtration, platelet concentrations were adjusted to 2.0×10⁸ platelets/ml with HBS. Aggregation studies were performed at 37°C using standard nephelometric technique as described (Freedman et al. 1996).

Platelet vitamin E content. Platelet tocopherol content was determined using HPLC with electrochemical detection. Briefly, GFP (0.5 ml) were extracted with an equal volume of methanol and 5 ml hexane. An aliquot of the hexane phase (4 ml) was dried under nitrogen, resuspended in ethanol (0.2 ml) and subjected to reverse-phase HPLC (Freedman et al. 1996, Keaney et al. 1996). The detection of RRR-α-tocopherol acetate was accomplished with UV detection at 286 nm.

Quantification of antioxidant capacity. LDL resistance to oxidation was used to determine the antioxidant capacity of the tocopherol isoforms as previously described (Shaery et al. 1997). LDL oxidative susceptibility was assessed by continuous spectrophotometric monitoring using a Varian Cary 3 spectrophotometer (Varian Co., Sugarland, TX). Conjugated diene generation (optical density, 234 nm) was assessed in LDL, and LDL susceptibility to lipid peroxidation was quantified by the length of the lag phase before the accelerated diene conjugation.

Phosphorylation of platelet proteins. PRP was incubated with 500 μmol/L vitamin E or vehicle at room temperature for 30 min followed by centrifugation at 800×g for 10 min to isolate platelets. Platelets were then loaded with [32P]orthophosphate (18.5 MBq/ml), and aggregation induced with 50 μmol/L arachidonic acid. Data are expressed as means ± SEM and are derived from three experiments; *P < 0.05 vs. vehicle treatment (not shown).

RESULTS

Effect of vitamin E on platelet aggregation. Loading platelets with RRR-α-tocopherol, RRR-α-tocopheryl acetate or synthetic α-tocopherol (all-rac-α-tocopherol) inhibited arachidonic acid–mediated platelet aggregation to distinct degrees (Fig. 1). Aggregation was inhibited by 57 and 52% with RRR-α-tocopherol and RRR-α-tocopheryl acetate, respectively, whereas all-rac-α-tocopherol produced no inhibition. The 50% inhibitory concentrations for platelet aggregation with RRR-α-tocopherol (385 ± 86 μmol/L) and RRR-α-tocopheryl acetate (392 ± 85 μmol/L) in response to arachidonic acid (50 μmol/L) were comparable.

Platelet incorporation of tocopherol isoforms. Incubation of PRP with RRR-α-tocopherol, RRR-α-tocopheryl acetate or all-rac-α-tocopherol for 30 min produced a dose-dependent increase in platelet incorporation of these compounds (Fig. 2A). The incorporation of tocopherol isoforms (500 μmol/L incubation) and the corresponding normalized extent of aggregation are shown in Figure 2B. Incorporation was greatest for RRR-α-tocopherol acetate compared with both RRR-α-tocopherol and all-rac-α-tocopherol. There was a decrease in platelet aggregation for RRR-α-tocopherol and RRR-α-tocopheryl acetate but not for all-rac-α-tocopherol. Incubation of

**FIGURE 2** α-Tocopherol isoform incorporation into platelets and platelet aggregation. Platelet-rich plasma (PRP) was incubated with 0–1 mmol/L RRR-α-tocopherol, RRR-α-tocopheryl acetate, all-rac-α-tocopherol or vehicle control for 30 min followed by gel-filtration, extraction and HPLC determination of platelet tocopherol content. (A) Incorporation of α-tocopherol isoforms into platelets. (B) Platelet α-tocopherol incorporation compared with the extent of platelet aggregation after incubation with 500 μmol/L of each isomer. Extent of platelet aggregation was measured after the addition of 50 μmol/L arachidonic acid. Data are expressed as means ± SEM and are derived from three experiments; *P < 0.05 vs. vehicle treatment (not shown).

**FIGURE 1** Representative tracing of α-tocopherol–mediated inhibition of in vitro platelet aggregation. Platelet-rich plasma (PRP) was incubated with 500 μmol/L RRR-α-tocopherol, RRR-α-tocopheryl acetate, all-rac-α-tocopherol or vehicle control for 30 min, gel-filtered, and aggregation induced with 50 μmol/L arachidonic acid. Data are representative of three independent experiments.
platelets with RRR-α-tocopherol acetate did not produce any increase in RRR-α-tocopherol acetate, indicating no esterase activity in plasma or platelets specific for this tocopherol derivative. Thus, minimal platelet incorporation may explain the lack of effect by the all-rac form of α-tocopherol.

Antioxidant activity of α-tocopherol isomers. We determined the lag phase for diene conjugation with 100 μmol/L of each tocopherol isomer using copper-mediated LDL oxidation (Fig. 3). We found no significant antioxidant effect of RRR-α-tocopherol acetate compared with vehicle, whereas RRR-α-tocopherol and all-rac-α-tocopherol were effective. Therefore, this experiment confirms that RRR-α-tocopherol acetate has minimal antioxidant capacity and suggests that inhibition of platelet aggregation does not require antioxidant activity. To confirm this notion, we compared the effect of RRR-α-tocopherol with its two-electron oxidation product, RRR-α-tocopherol quinone. As shown in Figure 4, RRR-α-tocopherol quinone was equal in potency to RRR-α-tocopherol.

Effect of α-tocopherol isomers on platelet PKC activity. We demonstrated previously that inhibition of platelet aggregation by RRR-α-tocopherol is PKC dependent. Therefore, we examined the effect of the different vitamin E isomers on platelet PKC stimulation. As shown in Figure 4, vehicle-treated platelets demonstrated phosphorylation of the 47-kDa PKC substrate in response to PMA; however, phosphorylation was prevented in platelets treated with RRR-α-tocopherol and RRR-α-tocopherol acetate. All-rac-α-tocopherol demonstrated no inhibition of phosphorylation.

DISCUSSION

The data presented here demonstrate that isomers of α-tocopherol produce variable inhibition of platelet aggregation. We found that RRR-α-tocopherol and RRR-α-tocopherol acetate, but not all-rac-α-tocopherol, led to dose-dependent inhibition of aggregation. The natural form of vitamin E, RRR-α-tocopherol, was the most effective in inhibiting platelet aggregation, particularly in response to PMA as demonstrated by near complete inhibition of phosphorylation of a 47-kDa PKC substrate (Fig. 5). However, RRR-α-tocopherol acetate and RRR-α-tocopherol quinone were also effective, even though they possess no antioxidant activity. Thus, these data indicate that the antioxidant activity of α-tocopherol is not required for inhibition of platelet aggregation.

In our previous study, we found that RRR-α-tocopherol is particularly effective in inhibiting platelet aggregation in response to PMA, an agonist that is dependent upon PKC stimulation (Freedman et al. 1996). This indirect evidence for PKC inhibition by α-tocopherol was confirmed by direct evidence from platelet protein phosphorylation studies demonstrating reduced phosphorylation of a 47-kDa PKC substrate in α-tocopherol–loaded platelets stimulated with PMA (Fig. 5). Moreover, here we demonstrate that the antioxidant activity of α-tocopherol is not required for PKC inhibition because RRR-α-tocopherol acetate was nearly as effective (Fig. 5). Thus, platelet inhibition by RRR-α-tocopherol is PKC dependent, and PKC inhibition stems from the nonantioxidant actions of vitamin E.

The precise mechanism of PKC inhibition by RRR-α-tocopherol is the subject of considerable investigation. Although not studied extensively in platelets, RRR-α-tocopherol inhibits PKC activity in cultured cells (Boscoboinik et al. 1991).
One putative mechanism for this effect is the activation of protein phosphatase 2a and dephosphorylation of PKCα (Ricciarelli et al. 1998). The precise molecular interactions that control this phenomenon are not known. From our data, one can imply that the activity of α-tocopherol in this regard does not likely involve the phenolic hydroxy group on the chromanol ring. Evidence to support this contention is derived from our observations that the antioxidant activity of α-tocopherol is not required for PKC inhibition (Figs. 4, 5) and the fact that blocking this group with an acetate ester also does not alter the activity of vitamin E. There is some evidence that the chromanol moiety of α-tocopherol does have a role, however, because β-tocopherol does not appear to be active (Boscoboinik et al. 1991, Ricciarelli et al. 1998) and differs from α-tocopherol only in substitution of the chromanol ring.

In summary, the data presented here demonstrate that distinct forms of α-tocopherol cause significant but variable inhibition of platelet aggregation, which does not correlate with antioxidant capacity. Differences were also observed for the extent of platelet incorporation as well as attenuation of PKC activity. Inhibition of platelet aggregation by vitamin E could explain in part its putative effect on coronary artery disease and its association with an increase in cerebral hemorrhagic risk (Tha Alpha Tocopherol Beta Carotene on the Incidence of Lung Cancer Prevention Study Group 1994).

**LITERATURE CITED**


