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EXPERIMENTAL STUDIES

Differential Effects of α- and γ-Tocopherol on Low-Density Lipoprotein Oxidation, Superoxide Activity, Platelet Aggregation and Arterial Thrombogenesis

Tom Saldeen, MD, PHD, FACC,† Dayuan Li, MD,* Jawahar L. Mehta, MD, PHD, FACC*
Gainesville, Florida and Uppsala, Sweden

OBJECTIVES This study was designed to examine the differential effects of α- and γ-tocopherol on parameters of oxidation-antioxidation and thrombogenesis.

BACKGROUND Experimental studies have shown that antioxidants, such as vitamin E (α-tocopherol), improve atherosclerotic plaque stability and vasomotor function, and decrease platelet aggregation and tendency to thrombus formation.

METHODS Sprague Dawley rats were fed chow mixed with α- or γ-tocopherol (100 mg/kg/day) for 10 days. A filter soaked in 29% $FeCl_3$ was applied around the abdominal aorta to study the patterns of arterial thrombosis. The aortic blood flow was observed and continuously recorded using an ultrasonic Doppler flow probe. ADP-induced platelet aggregation, low-density lipoprotein oxidation induced by phorbol 12-myristate 13-acetate (PMA)-stimulated leukocytes, superoxide anion generation and superoxide dismutase (SOD) activity were also measured.

RESULTS Both α- and γ-tocopherol decreased platelet aggregation and delayed time to occlusive thrombus (all $p < 0.05$ vs. control). Both α- and γ-tocopherol decreased arterial superoxide anion generation, lipid peroxidation and LDL oxidation (all $p < 0.05$ vs. control), and increased endogenous SOD activity ($p < 0.05$). The effects of γ-tocopherol were more potent than those of α-tocopherol ($p < 0.05$).

CONCLUSIONS This study indicates that both α- and γ-tocopherol decrease platelet aggregation and delay intraarterial thrombus formation, perhaps by an increase in endogenous antioxidant activity. γ-Tocopherol is significantly more potent than α-tocopherol in these effects. (J Am Coll Cardiol 1999;34:1208–15) © 1999 by the American College of Cardiology

Epidemiological studies (1–3) have provided evidence for an inverse relation between acute coronary events and antioxidant vitamin intake. Dietary supplementation with vitamin E, in particular, has been shown to reduce the number of ischemic cardiac events in patients with documented coronary artery disease (CAD) in one prospective study (4).

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There is evidence that plaque stability, vasomotor function, platelet aggregation and tendency to thrombosis can be modified by antioxidants (5). Antioxidants inhibit monocyte adhesion, protect against the cytotoxic effects of oxidized low-density lipoprotein (LDL) and reduce platelet activation (5). Vitamin E and antioxidant probucol also protect against endothelial dysfunction associated with atherosclerosis by preserving endothelium-derived nitric oxide (NO) activity (6,7). It is now generally recognized that platelet aggregation is abnormally increased in CAD patients (8,9). Experimental studies have shown that free radicals promote platelet aggregation and thrombosis (10,11) and chain breaking antioxidants, such as vitamin E, inhibit or delay arterial thrombogenesis (12).

NO, formed as a result of the action of constitutive NO synthase (cNOS) enzyme on L-arginine, is a powerful vasodilator and platelet aggregation inhibitor (13). Nitric oxide reacts with superoxide at near diffusion-limited rates to form peroxynitrite, another reactive oxygen intermediate, which can lead to the formation of hydroxyl and singlet

From the *Department of Medicine, University of Florida, and VA Medical Center, Gainesville, Florida, and the †Department of Forensic Medicine, University of Uppsala, Uppsala, Sweden.

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Abbreviations and Acronyms:

ANOVA = analysis of variance
CAD = coronary artery disease
cNOS = constitutive nitric oxide synthase
LDL = low density lipoprotein
MDA = malondialdehyde
NO = nitric oxide
SOD = superoxide dismutase
t-PA = tissue plasminogen activator

oxygen radicals (14). Vitamin E has been shown to enhance NO-dependent vasorelaxation (6) and decrease platelet aggregation (15), presumably related to inhibition of free radicals.

Vitamin E consists of two major different structural forms: α- and γ-tocopherol. In recent in vitro studies, γ-tocopherol has been shown to inhibit lipid peroxidative damage (16) and to trap mutagenic electrophiles (17) more efficiently than α-tocopherol. Further, it is the γ-tocopherol, and not α-tocopherol, that appears to be expressed in low amounts in the plasma of patients with CAD (18). A clinical study has shown that dietary supplementation with α-tocopherol, the primary form of vitamin E in commercial preparations, may further lower plasma γ-tocopherol levels (19). As such, dietary supplementation with α- and γ-tocopherol may have different biological effects.

Because of the potential differences in the antioxidant effect of α- and γ-tocopherol that may influence intravascular thrombus formation, we hypothesized that the two vitamin E formulations may have different effects on platelet aggregation and arterial thrombogenesis. This study was designed to explore these issues.

MATERIALS AND METHODS

Thirty-three male Sprague Dawley rats (weight 300 to 350 g) were used in this study. The rats were randomly fed regular rat chow mixed with rapeseed oil (800 mg/kg/day, n = 11), or chow and rapeseed oil mixed with α-tocopherol (Covitol F; Henkel, Dusseldorf, Germany), or γ-tocopherol (Cardinova, Uppsala, Sweden) (100 mg/kg/day each, n = 11 in each group) for 7 to 10 days. At the end of the feeding period, thrombogenesis was examined in all rats, and blood and aortic tissues were harvested for measurements described below. This study was approved by the appropriate Institutional Animal Care Committees.

Model of arterial thrombosis. An arterial thrombus model initially described by Kurtz et al. (20), and subsequently by us (21), was used in this study. In brief, animals were anesthetized with sodium pentobarbital (30 mg/kg). The abdominal cavity was opened and approximately 1.2 cm of the abdominal aorta was isolated. The aortic blood flow was observed for 1 h and continuously recorded using an ultrasonic Doppler flow probe (Crystal Biotech, Holliston, MA).

Whatman paper soaked in 29% $FeCl_3$ was wrapped around the external surface of the aorta. After the thrombus was formed, the exposed aorta was removed and the thrombus was weighed. Blood was collected for measuring plasma α- and γ-tocopherol levels, superoxide dismutase (SOD) activity and determination of platelet aggregation. The abdominal aorta proximal to the thrombus was saved for measurement of superoxide anion generation, lipid peroxidative product malondialdehyde (MDA) and SOD activity. Leukocytes from five normal rats on regular chow were used to study oxidation of LDL.

Measurement of α- and γ-tocopherol in plasma. Plasma levels of α- and γ-tocopherol were measured by high-performance liquid chromatography with fluorescence detection, as described earlier (22).

Platelet aggregation. Blood was gently mixed with 3.8% sodium citrate (9:1), centrifuged at 1,200 rpm for 10 min at room temperature to obtain platelet-rich plasma (PRP) and centrifuged again at 3,000 rpm for 15 min to obtain platelet-poor plasma. Platelet count in PRP was counted and kept at about 2 to 3 × 10^8cells/ml. ADP (final concentration 20 μM) was used as stimulus for platelet aggregation. This concentration of ADP has been used in several experiments in our laboratory (12,21,23). All aggregations were conducted in a four-channel aggregometer (BIO/DATA, Horsham, Pennsylvania) in duplicate.

Determination of SOD activity. SOD activity in plasma and aortic homogenates, as an index of endogenous antioxidant activity, was measured spectrophotometrically in duplicate by monitoring the SOD-inhabitable autooxidation of pyrogallol, as described in our previous study (24). The reaction mixture (4.5 ml) consisted of 0.2 mmol/L of pyrogallol, 1 mmol/L of diethylenetriamine penta-acetic acid, 50 mmol/L of *tris*-cacodylic acid buffer (pH 8.2) and 4 μg of catalase. The reaction was carried out at 25°C. The rate of increase in absorbance at 420 nm was recorded. One unit of enzyme activity is defined as 50% inhibition of pyrogallol autooxidation under the assay conditions. The SOD activity was expressed as units per milliliter in plasma and units per milligram protein in aortic homogenates.

Arterial superoxide anion generation. An approximately 0.6-cm segment of abdominal aorta proximal to the thrombus was excised, and placed in oxygen-saturated Krebs-Ringer buffer, cleaned of fat and loose connective tissue. Care was taken to avoid any unnecessary manipulation of the vessel. The rate of superoxide anion formation by abdominal aortic segments was determined by chemiluminescence of lucigenin (bis-*N*-methyl-acridinium nitrate), as described recently (25). Briefly, Krebs-Ringer buffer containing 0.25 mM lucigenin (pH 7.4) was prepared as an assay solution. One milliliter of this assay solution was placed in a glass scintillation vial, and then an aortic

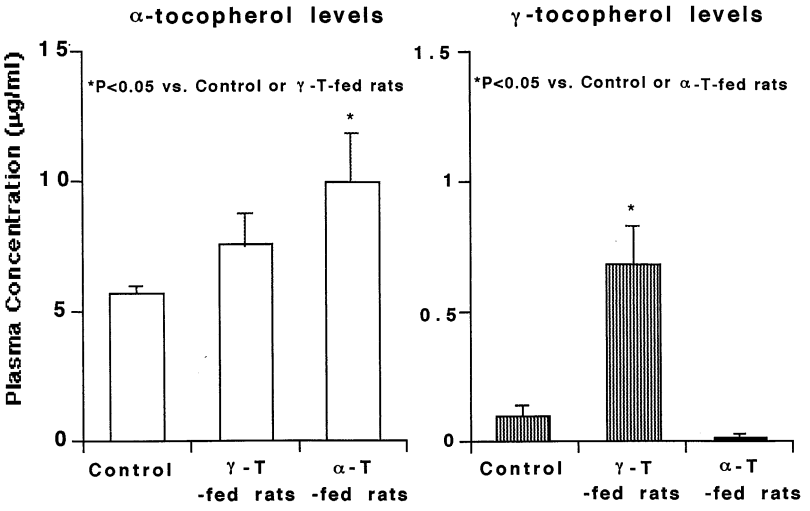

Figure 1. Plasma levels of α- and γ-tocopherol in rats fed different diets. Plasma α-tocopherol levels increased markedly in α-tocopherol-fed rats, and γ-tocopherol levels decreased as compared with the control rats. On the other hand, plasma γ-tocopherol levels were markedly elevated in γ-tocopherol-fed rats, and α-tocopherol levels were not different as compared with control rats. α-T = α-tocopherol; γ-T = γ-tocopherol. Data are mean ± SD (n = 11 rats in each group).

segment was gently placed in the assay solution. The chemiluminescence of lucigenin was then detected with the use of a scintillation counter (LS 7000; Beckman, Irvine, California) in out-of-coincidence mode with a single active photomultiplier tube every 3 min. The chemical specificity of this light-yielding reaction for superoxide anion has been reported previously, and the sensitivity and specificity of this assay was determined with xanthine (100 to 400 nM) and xanthine (0.002 U) to generate superoxide anion with or without SOD (25). The data on superoxide anion generation was expressed as counts per minute per milligram (cpm/mg).

Determination of lipid peroxidation (MDA). MDA formation was measured in the plasma as index of lipid peroxidation, as described earlier (24). The assay mixture consisted of 0.1 ml of plasma or supernatant of aortic homogenate, 0.4 ml of 0.9% NaCl, 0.5 ml of 3% sodium dodecylsulfate, 3 ml of thiobarbituric acid reagent (containing equal parts of 0.8% aqueous thiobarbituric acid and acetic acid), and was heated for 75 min at 95°C. Thereafter, 1 ml cold 0.9% NaCl was added to the mixture, which was cooled and extracted with 5 ml n-butanol. After centrifugation at 3,000 rpm for 15 min, the butanol phase was assayed spectrophotometrically at 532 nm. MDA (in amounts of 0, 0.1, 0.2, 0.4, 0.8, and 1 nmol) served as external standard. Malondialdehyde content in the plasma was expressed as nanomoles per milliliter.

α- and γ-tocopherol and LDL oxidation. To examine the effect of α- and γ-tocopherol on LDL oxidation, native LDL (50 μg/ml) was incubated with rat leukocytes (10^6 cells/ml) and phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) in 2 ml of Tyrode's buffer (mmol/L:NaCl 137, KCl 2.7, $MgCl_2$ 1.0, $CaCl_2$ 1.0, NaH_2PO_4 0.35, $NaHCO_3$ 11.9 and glucose 5.5, pH 6.5) with or without 100 μl of plasma from rats fed regular diet or α- and γ-tocopherol-rich diet. After 1 h of incubation at 37°C, kinetics of LDL oxidation in the supernate were determined by monitoring the change in absorbance (234 nm) in an ultraviolet spectrophotometer (Shimadzu, Kyoto, Japan). Absorbance was recorded every hour for 6 h. From the kinetic absorbance profile of each experiment, the rate of oxidation was calculated from the slope of absorbance curve during the propagation phase, expressed as nmoles dienes/min per mg LDL protein (22). The methods for isolation of leukocytes and native LDL have been described earlier (22).

Statistical analysis. All data represent the mean of duplicate samples from at least five independently performed experiments. Data are presented as mean ± SD. Statistical significance in multiple comparisons was determined among independent groups of data in which analysis of variance (ANOVA) followed by Fisher's exact test indicated the presence of significant differences. A p value < 0.05 was considered significant.

RESULTS

Plasma α- and γ-tocopherol levels. Plasma α-tocopherol levels were markedly higher in α-tocopherol-fed rats ($p < 0.01$ as compared with control or γ-tocopherol-fed rats). The γ-tocopherol levels were very low in the control group of rats and decreased further in the α-tocopherol-fed rats. On the other hand, plasma γ-tocopherol levels were markedly elevated in γ-tocopherol-fed rats, while α-tocopherol levels in these rats were comparable with the control rats (Fig. 1).

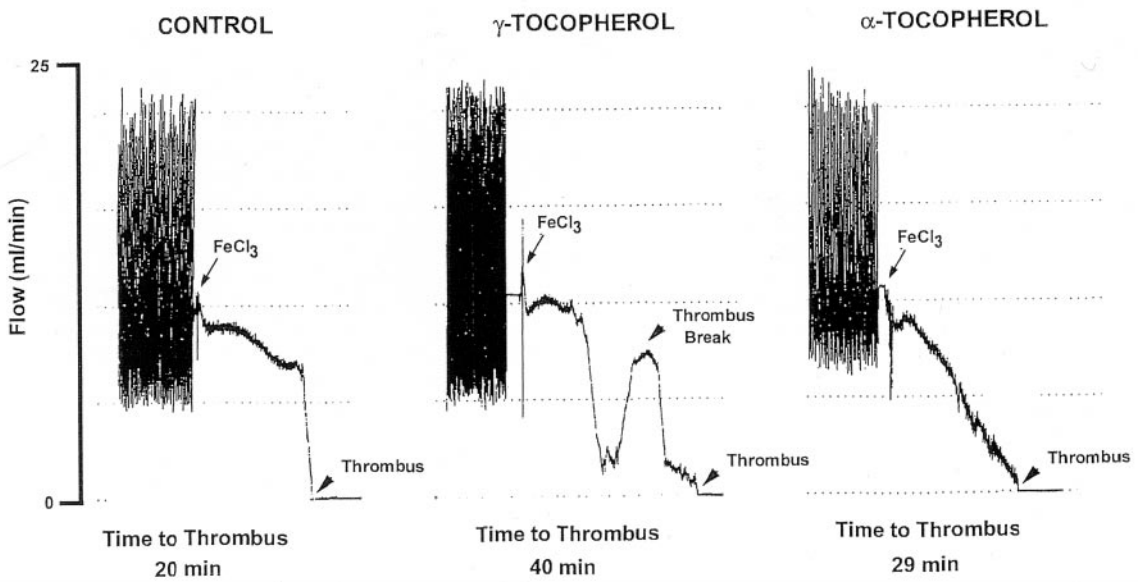

Figure 2. Representative examples of thrombus formation in rats from control group, α-tocopherol and γ-tocopherol group. The time to thrombus formation, as indicated by cessation of flow, is 9 min longer in α-tocopherol fed rat, whereas thrombus formation is delayed markedly in the γ-tocopherol group. Please note the initial decrease in aortic flow in the rat from the γ-tocopherol group suggesting platelet aggregation; however, the flow is quickly restored suggesting break of the platelet aggregates.

Time to thrombosis and platelet aggregation. Application of $FeCl_3$ in control rats resulted in oscillations in aortic blood flow for about 15 to 20 min. This was followed by a rapid decrease in blood flow and eventually total cessation, indicating occlusive thrombus formation. During the 60-min period of observation, there was no evidence of return of flow in any of the control animals.

In rats fed α-tocopherol, the time to thrombus formation was increased 25% ($p < 0.05$ as compared with control rats). In rats fed γ-tocopherol, thrombus could not be formed over the entire 1-h period of observation in one animal, and the time to thrombus formation was 58% longer in the remaining 10 rats than in control rats ($p < 0.01$). The time to thrombus formation was longer in γ-tocopherol-fed rats than in the α-tocopherol-fed rats ($p < 0.05$). In contrast to stable thrombus in the control rats, the thrombus was unstable in three of 11 α-tocopherol-fed rats, as indicated by spontaneous return of blood flow during the period of observation. The thrombus was unstable in 6 of 10 γ-tocopherol-fed rats, as indicated by spontaneous return of blood flow during the one-hour period of observation. Representative patterns of thrombus formation in the three groups of rats are shown in Figure 2, and the summary of data on thrombogenesis in all animals are presented in Figure 3.

Platelet aggregation was less in rats fed α- or γ-tocopherol ($p < 0.05$ as compared with control rats). The inhibition of platelet aggregation was greater in γ-tocopherol-fed rats than in the α-tocopherol-fed rats ($p < 0.05$) (Fig. 3).

Oxidation of LDL. Plasma from both α- or γ-tocopherol-fed rats significantly decreased the rate of LDL oxidation induced by PMA-stimulated leukocytes ($p < 0.05$ vs. control rats). γ-Tocopherol was significantly more potent in reducing oxidizability of LDL than was α-tocopherol ($p < 0.05$) (Fig. 4).

Superoxide anion generation and lipid peroxidation. Arterial superoxide anion generation was 19,800 ± 2,400 cpm/mg in the control rats. This value was reduced by 28% in the α-tocopherol-fed rats and by 49% in the γ-tocopherol-fed rats (both $p < 0.05$ vs. control rats). The superoxide anion generation in the γ-tocopherol-fed rats was markedly less than in α-tocopherol-fed rats (Fig. 5).

Plasma MDA, a lipid peroxidation product, was also decreased in both α- or γ-tocopherol-fed rats ($p < 0.05$ vs. control rats). Plasma MDA in the γ-tocopherol-fed rats was less than that in the α-tocopherol-fed rats ($p < 0.05$) (Fig. 5).

SOD activity. Administration of α- or γ-tocopherol significantly increased SOD activity in aortic homogenates as well as in plasma ($p < 0.05$ vs. control rats). Feeding of γ-tocopherol to rats caused greater effect on SOD activity than feeding of α-tocopherol ($p < 0.05$) (Fig. 6).

DISCUSSION

This study demonstrates that both α- and γ-tocopherol significantly decrease platelet aggregation and delay the time to occlusive thrombus formation. Both α- and γ-tocopherol markedly decrease arterial superoxide anion generation, lipid peroxidation and LDL oxidation. Interestingly, both α- and γ-tocopherol also increase endogenous SOD activity in different tissues. Importantly, we found that γ-tocopherol was more potent than α-tocopherol in all these effects.

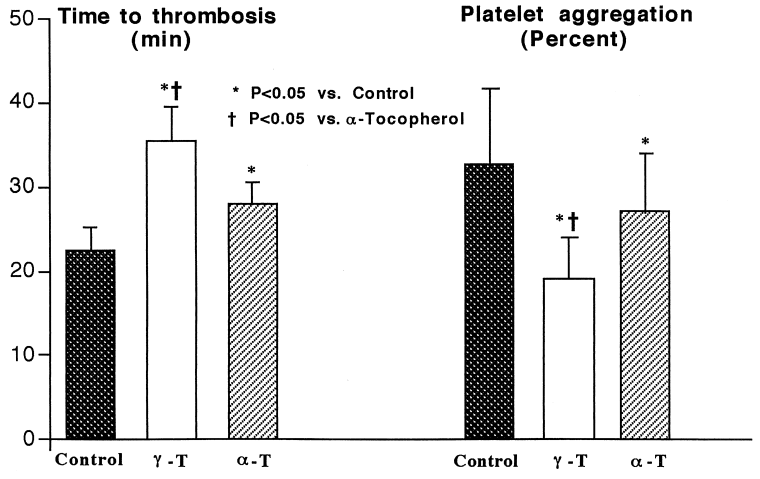

Figure 3. Summary of data on time to thrombus formation and platelet aggregation in rats from control group, α-tocopherol and γ-tocopherol group. Data (mean ± SD) shown are from 11 rats in the control group, 11 rats in the α-tocopherol group and 10 rats in the γ-tocopherol group

Before discussing these results, a comment needs to be made relative to the thrombus model used in this study. In this rat model, the thrombus is formed rapidly (15 to 25 min) in response to application of $FeCl_3$, and is characterized by endothelial disruption, extensive platelet and red blood cell clumps and interspersed fibrin (20,21). This morphology of the thrombus is similar to the thrombi in the coronary arteries of patients with acute myocardial ischemia (26). The low cost of the animals, ease of thrombus formation and similarity to human thrombus make the rat model unique to the study of influence of different agents that can inhibit platelet aggregation and thrombosis. $FeCl_3$ causes oxidative injury to the endothelium, which facilitates platelet deposition to the subendothelial layers, and the effects of vitamin E may reflect its potent antioxidant effect. Similar antithrombotic effects of vitamin E have been shown in non-$FeCl_3$-induced thrombosis models (10,11).

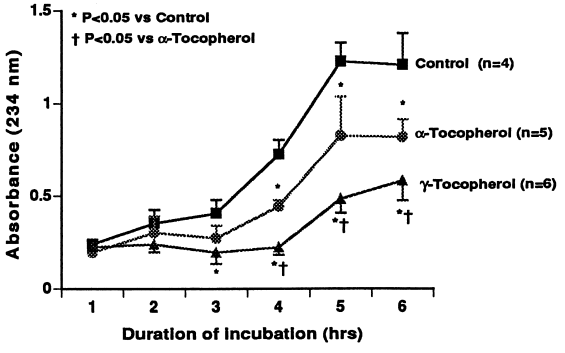

Figure 4. Oxidation of LDL by PMA-stimulated leukocytes in the presence of rat plasma. Administration of α- or γ-tocopherol to rats significantly decreased the rate of oxidation induced by PMA-stimulated leukocytes ($p < 0.05$ vs. control rats). γ-Tocopherol had stronger effect in reducing oxidizability of LDL than α-tocopherol ($p < 0.05$). Data (mean ± SD) shown are from five separate experiments.

Antioxidant vitamins and thrombosis. Clinical and pathological studies have shown that disruption or erosion of atherosclerotic plaque is the major cause of coronary thrombosis, which is the basis of precipitation of acute coronary syndromes (8,9). Atherosclerosis and thrombosis are associated with many common pathological features, such as deposition and aggregation of platelets, monocyte/macrophage infiltration and dysfunctional endothelium. Furthermore, both these conditions are associated with increased oxidative stress (8,27). Atherosclerotic lesion formation is associated with accumulation of lipid peroxidation products and the induction of inflammatory genes by NF-κB, a redox-sensitive transcription factor (28). Vitamin E and other antioxidants have been shown to reduce thrombus formation (10,12), extent of atherosclerosis in cholesterol-fed animals (23) and the incidence of acute cardiac events in men and women (1–3). The impaired endothelium-dependent relaxation accompanying the atherosclerotic process can be restored with administration of vitamin E and the antioxidant probucol (6,7). Recent studies show that vitamin E inhibits platelet aggregation by a protein kinase C-dependent mechanism (15). In the present study, we demonstrate that both α- and γ-tocopherol significantly inhibit platelet aggregation and delay thrombus formation in the rat. Interestingly, we found that both α- and γ-tocopherol affect the stability of arterial thrombus, suggesting an increase in the endogenous thrombolytic potential by both vitamin E preparations. It is possible that inhibition of platelet aggregation and thrombosis is the major mechanism by which vitamin E decreases the frequency of acute cardiac events in patients with preexisting CAD (4).

α- and γ-tocopherol, free radicals and thrombosis. Numerous experimental studies suggest a major role for oxygen-free radicals in the pathogenesis of the myocardial

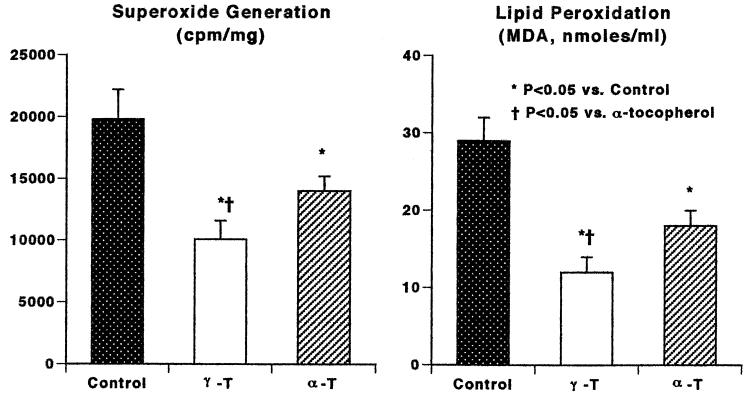

Figure 5. Summary of data on arterial superoxide generation and plasma MDA levels. Data (mean ± SD) shown are from 11 rats in the control group, 11 rats in the α-tocopherol group and 11 rats in the γ-tocopherol group

lesions observed during the process of ischemia-reperfusion. Free radicals promote platelet aggregation, injure endothelium and enhance intravascular thrombus formation (10–12,29). Jourdan et al. (11) showed induction of thrombus by free radicals in rats and an antithrombotic effect of vitamin E on thrombus formation induced by free radicals. An earlier study from our laboratory (30) showed that the antioxidant SOD when administered before tissue plasminogen activator (t-PA) prevented coronary artery reocclusion after thrombolysis in a canine model of coronary artery thrombosis. Ikeda et al. (10) found that SOD plus catalase reduced cyclic flow variations in narrowed coronary arteries of dog coronary artery. These cyclic flow reductions are thought to be caused by platelet aggregation occurring in blood vessels with dysfunctional endothelium. Przyklenk and Kloner (31) also showed that administration of antioxidants inhibits platelet aggregation and promotes thrombolytic effect of t-PA. Nunes et al. (32) showed that neointimal proliferation after balloon angioplasty of pig coronary arteries is accompanied by an increased production of superoxide anions, apparently by smooth muscle cells and fibroblasts in the vessel wall. This increase in superoxide anions was attenuated when pigs were pretreated with vitamin C, vitamin E or their combination for seven days before angioplasty. These data collectively indicate that reactive oxygen species released from vessel wall and blood constituents cause platelet aggregation, intimal proliferation and thrombosis. In the present study, we found that both α- and γ-tocopherol significantly reduced superoxide anion generation and lipid peroxidation product MDA. These data may have implications relative to improvement in endothelial function, reduction in platelet aggregation and inhibition of thrombosis after therapy with vitamin E.

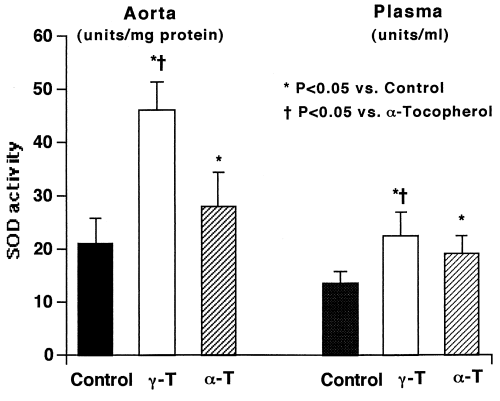

Figure 6. SOD activity in aortic homogenates and plasma from rats fed different diets. Feeding of rats with α- and γ-tocopherol increased SOD activity in the supernatants of aortic homogenates and in the plasma. Data (mean ± SD) shown are from 11 rats in the control group, 11 rats in the α-tocopherol group and 11 rats in the γ-tocopherol group.

α- and γ-tocopherol and SOD activity. In two recent studies, one in rats fed a high-fructose diet (33) and the other in rabbits given a high-cholesterol diet (22), administration of vitamin E was associated with an increase in SOD activity. In another study (34), Martin et al. found that vitamin E supplementation significantly increases SOD and catalase activity in aortic endothelial cells. In the current study, we demonstrate that both α- and γ-tocopherol significantly increase SOD activity in plasma as well as in the aortic tissues. These findings may also have relevance in inhibition of platelet aggregation, prolongation of time to thrombosis and instability of the thrombus. This hypothesis is consistent with the well-known effects of SOD on platelet aggregation and thrombogenesis (10,30,31).

Antioxidant vitamins and atherosclerosis. The oxidative-modification hypothesis implies that it is the oxidatively modified LDL that is relevant in atherogenesis (5). There is evidence that plaque stability, vasomotor function and tendency to thrombosis are subject to modification by specific antioxidants. For example, cellular antioxidants inhibit monocyte adhesion, protect against the cytotoxic effects of oxidized-LDL and inhibit platelet activation. It is widely appreciated that LDL, especially its oxidatively modified form, oxidized-LDL, is a critical factor in athero-

genesis and platelet aggregation (35,36). Ox-LDL has also been implicated in the pathogenesis of acute myocardial infarction (37). The endothelial dysfunction and increased platelet aggregation are present before and throughout the development of atherosclerosis and particularly during plaque rupture. Studies (24,35) from our laboratory have shown that ox-LDL causes human coronary artery endothelial cell injury (by apoptosis and necrosis) as well as platelet aggregation (by inhibition of NO synthase activity). In the current study, we used PMA to induce leukocytes to release free radicals, and the latter causes LDL oxidation. We found that plasma from α and γ-tocopherol-fed rats significantly reduced oxidation of LDL. Additional evidence for the antioxidant effect of α- and γ-tocopherol came from reduction in MDA levels in plasma of rats. These potent antioxidant effects may underlie the attenuation of thrombus formation by vitamin E. These observations collectively suggest that the protective effects of α- and γ-tocopherol against progression of atherosclerosis and thrombus formation may relate to reduction of LDL oxidation.

Greater efficacy of γ-tocopherol than α-tocopherol. In this study, we demonstrate that γ-tocopherol is significantly more potent than α-tocopherol in inhibiting platelet aggregation and thrombogenesis. We also found that γ-tocopherol reduced superoxide anion generation, lipid peroxidation and LDL oxidation, and increased SOD activity to a much greater extent than α-tocopherol.

The harmful effect of free radicals is best counteracted by γ-tocopherol and to a lesser extent by α-tocopherol (16,17). An in vitro study (38) has even suggested that α-tocopherol may become prooxidant in large concentrations; however, we did not observe any evidence of prooxidant effect of feeding α-tocopherol to the rats. Several pieces of evidence suggest that γ-tocopherol, when taken as part of diet, may be more important than α-tocopherol in the prevention of CAD-associated morbidity (3). Incidentally, it is the γ-tocopherol levels that are low in plasma, whereas α-tocopherol levels are in the normal range, in CAD patients (18).

Summary. This study shows that both α- and γ-tocopherol inhibit platelet aggregation and delay arterial thrombus formation in rats. Dietary supplementation with α- and γ-tocopherol in rats also reduced superoxide generation, lipid peroxidation and LDL oxidation, and increased SOD activity. Importantly, γ-tocopherol was more potent than α-tocopherol in all these effects. The clinical implications of these findings are enormous in terms of recommending supplementation of commercial antioxidant preparations with γ-tocopherol to patients with vascular disease or those at high risk of developing it.

Reprint requests and correspondence: Dr. J.L. Mehta, Department of Medicine, University of Florida College of Medicine, 1600 Archer Road, P.O. Box 100277, JHMHC, Gainesville, Florida 32610.E-mail:mehta@medmac.ufl.edu.

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