J Cardiovasc Pharmacol Therapeut 6(2):155–161, 2001

Different Isoforms of Tocopherols Enhance Nitric Oxide Synthase Phosphorylation and Inhibit Human Platelet Aggregation and Lipid Peroxidation: Implications in Therapy with Vitamin E

Dayuan Li, MD, PhD, Tom Saldeen, MD, PhD,* Francesco Romeo, MD,† and Jawahar L. Mehta, MD, PhD

Background: α-Tocopherol has received much attention in the primary and secondary prevention of coronary artery disease. Absence of other isoforms, such as γ- and δ-tocopherol, in commercial preparations of vitamin E may account for the inconsistent results of clinical trials. Since platelet aggregation is intimately involved in thrombogenesis, the relative effects of α-, γ-, and δ-tocopherol and their combination were examined on human platelet aggregation, lipid peroxidation, and constitutive nitric oxide synthase (cNOS) activity.

Methods and Results: Human platelets were incubated with the three different isoforms of tocopherol and their combination for 30 minutes, and then ADP-induced platelet aggregation measured. All three isoforms of tocopherol markedly and similarly decreased platelet aggregation in a concentration (120–480 μM)-dependent manner. All three tocopherols also decreased the level of the lipid peroxidation product, malondialdehyde (MDA), and increased NO release ($P < 0.05$ vs control). These isoforms of tocopherol did not affect cNOS protein expression, but enhanced cNOS phosphorylation in platelets. The combination of three tocopherols in a concentration found in nature was more potent than α-, γ-, or δ-tocopherol alone in this regard.

Conclusion: These observations suggest that all three major isoforms of tocopherol have a similar effect on human platelet aggregation. The three isoforms appear to attenuate platelet aggregation at least in part via a decrease in free radical generation and an increase in platelet cNOS activity. The combination of tocopherols has a synergistic platelet inhibitory effect. Future clinical trials should concentrate on the combination of these three isoforms of tocopherols.

Key words: human platelets, tocopherols, free radicals, nitric oxide synthase.

Increasing evidence shows that plaque stability, vasomotor function, platelet aggregation and tendency to thrombosis can be modified by antioxidants (1). Antioxidants inhibit monocyte adhesion, protect against the cytotoxic effects of oxidized LDL, and reduce platelet activation (1). Vitamin E and synthetic antioxidants also protect against endothelial dysfunction associated with atherosclerosis by preserving endothelium-derived nitric oxide (NO) activity (2,3). It is now generally recognized that platelet aggregation is abnormally increased in patients with coronary artery disease (CAD) (4,5). Experimental studies have also shown that free radicals promote platelet aggregation and thrombosis (6,7), and chain-breaking antioxidants, such as vitamin E, inhibit or delay arterial thrombogenesis (8).

Numerous studies have demonstrated that commercial vitamin E (α-tocopherol) has an important antioxidant effect. However, results of clinical trials with

*From the Department of Medicine, University of Florida and VA Medical Center, Gainesville; the *Department of Surgery, University of Uppsala, Uppsala, Sweden; and the †Department of Cardiology, University of Rome "Tor Vergata," Rome, Italy.*

Supported by a Merit Review Award from the VA Central Office, a grant from the Department of Defense, and financial support from the Swedish Medical Research Council, Stockholm, Sweden.

Reprint requests: J. L. Mehta, MD, PhD, The Stebbins Chair in Cardiology, Chairman, Division of Cardiovascular Disease, Professor of Internal Medicine and Physiology, Associate Chairman, Department of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR 72205-7199.

commercial vitamin E preparations (α-tocopherol) have yielded conflicting results (2,3,8–10). On the other hand, a natural source of vitamin E may be more beneficial; this approach has been advocated by the American Heart Association (10). Natural vitamin E consists of four different structural forms: α-, β-, γ-, and δ-tocopherol. In vitro studies show that γ-tocopherol, found in large amounts in natural sources of vitamin E, inhibits lipid peroxidative damage (11) and traps mutagenic electrophiles (12) more efficiently than does α-tocopherol.

Recent studies from our laboratory (13,14) showed that both α-tocopherol and γ-tocopherol–rich preparations decrease platelet aggregation and delay intraarterial thrombus formation by an increase in endogenous superoxide dismutase (SOD) and constitutive nitric oxide synthase (cNOS) expression and activity. These studies also suggested that γ-tocopherol–rich preparation is more potent than α-tocopherol in inhibiting lipid peroxidation and platelet-dependent thrombus formation. Since platelet aggregation may be crucial in conversion of acute to chronic CAD, this study was designed to examine the relative effects of α-, γ-, and δ-tocopherol isoforms on human platelet aggregation. We did not examine the effect of β-tocopherol in this study because the level of β-tocopherol is very low in nature (15).

Materials and Methods

Preparation of Platelets and Platelet Aggregation

Peripheral human venous blood from normal healthy volunteers who had not taken any drugs in the previous 10 days was gently mixed with 3.8% sodium citrate (9:1). The blood was centrifuged at 800 rpm for 10 minutes at room temperature to obtain platelet-rich plasma (PRP), and centrifuged again at 3,000 rpm for 15 minutes to obtain platelet-poor plasma. The platelet count in the PRP was kept at about 3×10^8 cells/mL. Adenosine diphosphate (ADP, final concentration 5 μM) was used as the stimulus for platelet aggregation. This concentration of ADP has been used previously in our laboratory (16,17). All aggregation studies were conducted in a four-channel Chronolog aggregometer in duplicate.

Experimental Protocol

α-, γ-, δ-Tocopherol and their combination were dissolved in 100% ethanol. The final volume of ethanol in PRP was less than 5%. The PRP was incubated with α-, γ-, δ-, and their combination (f.c. 120, 240, 360, 480 μM) for 30 minutes at 37°C before initiating platelet aggregation. Control PRP was incubated with an equal amount of ethanol before aggregation with ADP. After 5 minutes of aggregation, PRP was centrifuged, the platelet pellet was saved to measure tocopherol levels, and the supernatant was collected to measure NO and lipid peroxidation product malondialdehyde (MDA).

α-, γ-, and δ-Tocopherol were obtained from Sigma, St. Louis. Each preparation was over 98% pure. A combination of tocopherols (Cardi-E, Cardinova, Uppsala, Sweden) contained 63% γ-tocopherol, 25% δ-tocopherol, and 12% α-tocopherol. This natural vitamin E preparation, derived from vegetable oils as well as soybean, canola, sunflower, peanut, cottonseed, palm or coconut oil (15), did not contain a significant amount of β-tocopherol.

Measurement of α-, γ-, and δ-Tocopherols in Platelets

α-, γ-, δ-tocopherol levels in platelets were measured by high-performance liquid chromatography with fluorescence detection as previously described (16).

Determination of NO Release

A colorimetric NO assay kit was purchased from Oxford Co. This kit employs immunoaffinity purified nitrate reductase to measure total NO produced following enzymatic conversion to nitrite. The procedures followed were the same as those described by the manufacturer. Different amounts (0, 10, 20, 50, 100, 200, 400 μM) of nitrite served as the external standard. Nitrite was measured in the supernatants of platelets at 540 nm and expressed as micromoles per milliliter (17).

Immunoprecipitation and Western Blot for Activation of cNOS in Platelets

Activation of cNOS in platelets was determined by measurement of its phosphorylation. For identification of cNOS phosphorylation, platelet lysates were subjected to immunoprecipitation and then Western analysis. In brief, platelet lysates containing equal amounts of soluble proteins were immunoprecipitated with a monoclonal antibody to human cNOS (Santa Cruz Lab). Precipitates were washed and then resuspended in SDS-PAGE sample buffer and boiled for 5 minutes. Samples were separated by 8% SDS-PAGE, and then transferred to nitrocellulose membranes. After incubation in blocking solution (4% non-fat milk, Sigma), membranes were incubated with a poly-

clonal antibody to serine/threonine phosphorylation (Santa Cruz Lab) overnight at 4°C. The membranes were washed and then incubated with a 1:3000 dilution of secondary antibody (Amersham Life Science) for 1 hr, and detected by the ECL system. A western blot for cNOS was also performed as previously described (18,19).

Determination of Lipid Peroxidation

Malondialdehyde formation, which served as an index of lipid peroxidation, was measured in the supernatants of aggregated platelets as previously described (18,19); MDA content in platelet supernatant was expressed as nanomoles per milliliter.

Statistical Analysis

All data represent the mean of duplicate samples from at least six independently performed experiments in each group. Data are presented as mean ±SD. Statistical significance in multiple comparisons was determined among independent groups of data in which ANOVA and the F test indicated the presence of significant differences. A P value < 0.05 was considered significant.

Results

Platelet α-, γ-, and δ-Tocopherol Levels

Platelet α-, γ-, δ-tocopherol levels were very low in platelets from normal healthy subjects. In contrast, incubation of platelets with different concentrations of α-, γ-, δ-tocopherols increased tocopherol levels in platelets in a concentration-dependent manner. Data from incubation of PRP with 480 μM of α-, γ-, δ-tocopherol, or their combination are shown in Fig. 1.

Tocopherols and Platelet Aggregation

In the control group, the presence of 1 to 4 μL of ethanol did not affect platelet aggregation. α-, γ-, and δ-Tocopherols significantly inhibited platelet aggregation (all $P < 0.05$ vs control, n = 10 each). The inhibition of aggregation appeared to be a concentration-dependent phenomenon. Each isoform of tocopherol exerted a similar inhibitory effect in platelet aggregation. Importantly, we found that the combination of three tocopherols caused a greater inhibitory effect on platelet aggregation compared with each separate isoform (Fig. 2).

NO Release and cNOS Activity

Nitric oxide release was markedly increased in the supernatant of α-, γ-, δ-tocopherol–treated platelets ($P < 0.01$ vs control group, n = 10 each group). Nitric oxide in the supernatant of platelets incubated with the combination of three tocopherols further increased, as compared with the NO release in the supernatant of α-, γ-, or δ-tocopherol-treated platelets ($P < 0.05$) (Fig. 3).

Whereas cNOS protein expression was unaffected by tocopherols, cNOS activity was markedly increased in α-, γ-, or δ-tocopherol–treated platelets compared with control platelets. cNOS activity in platelets incubated with the combination of three tocopherols increased further, as compared with α-, γ-, δ-tocopherol–treated platelets alone (Fig. 4).

Lipid Peroxidation

Levels of MDA in the supernatant of α-, γ-, δ-tocopherol–treated aggregated platelets ($P < 0.01$ vs control group, n = 10 each group) were decreased. Malondialdehyde levels in the supernatant of aggregated platelets incubated with the combination of three tocopherols decreased further as compared with MDA levels in the supernatants of α-, γ-, or δ-tocopherol–treated platelets ($P < 0.01$) (Fig. 5).

Discussion

This study demonstrates that the three isoforms α-, γ-, and δ-tocopherol have similar inhibitory effects on

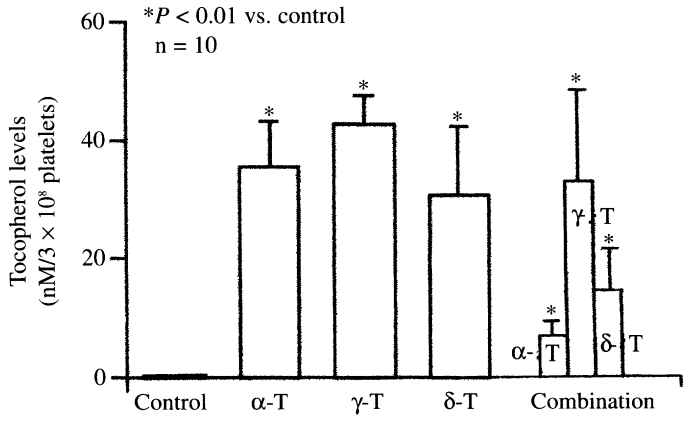

Fig. 1. α-, γ-, and δ-tocopherol levels in human platelets. The levels of α-, γ-, and δ-tocopherol and their combination were increased in platelets after incubation of platelets for 30 minutes. The concentration of tocopherols in this illustration was 480 μM. Data are mean ±SD (n = 10).

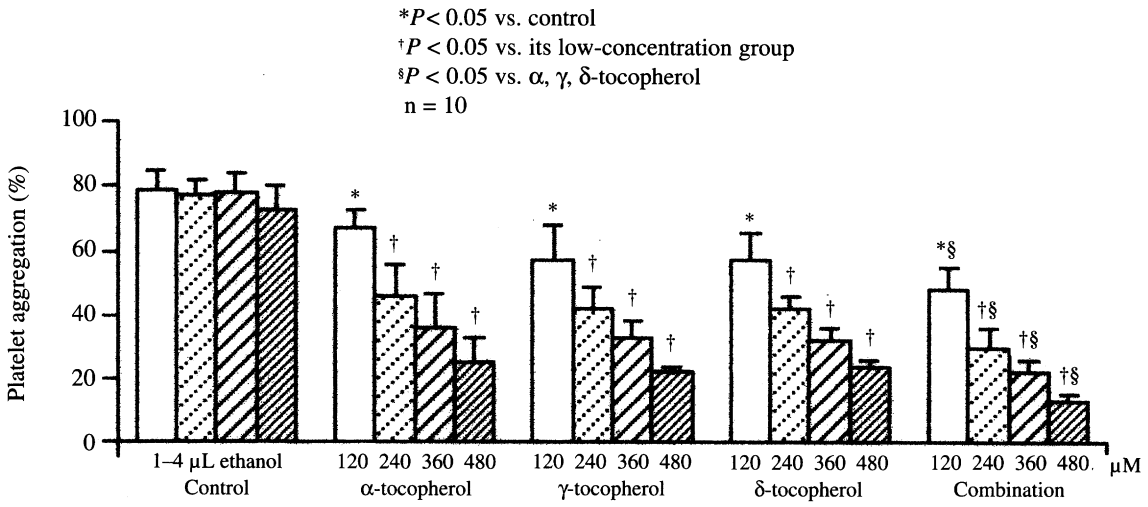

Fig. 2. Summary of data on platelet aggregation from each group. Incubation of PRP with α-, γ-, and δ-tocopherol had similar inhibitory effect on ADP-induced platelet aggregation. The combination of three tocopherols had more powerful effect on platelet aggregation compared with α-, γ-, or δ-tocopherol alone ($P < 0.05$). Data are mean ±SD (n = 10).

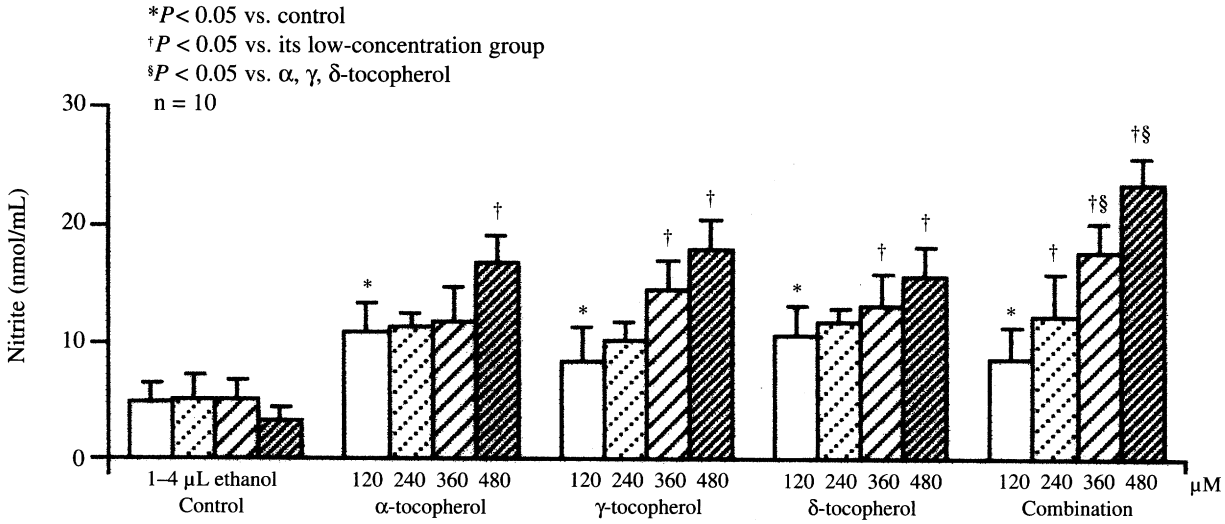

Fig. 3. The effect of tocopherols on NO release. Incubation of PRP with α-, γ-, and δ-tocopherols increased NO release compared with the control group ($P < 0.05$). The combination of three tocopherols further increased NO release compared with α-, γ-, or δ-tocopherol alone ($P < 0.05$).

platelet aggregation and the combination of α-, γ-, and δ-tocopherol, as found in natural foods, has a greater inhibitory effect on platelet aggregation than each individual isoform. Importantly, the inhibitory effect of different tocopherols on platelet aggregation is associated with a decrease in lipid peroxidation and an increase in cNOS activity.

The biological activity of different tocopherols and its definition differs markedly. One milligram of *d*-α-tocopherol has been defined as 1 α-tocopherol equivalent (α-TE), whereas 1 milligram of *d*-γ-tocopherol is only 0.1 α-TE, and 1 mg of *d*-δ-tocopherol is believed to have no equivalency (20). These official values are based on anti-sterility activity in rats. This official def-

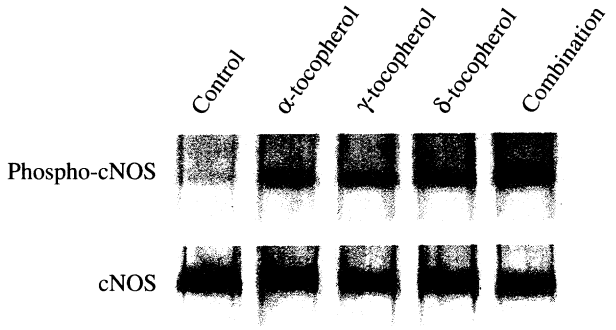

Fig. 4. The effect of tocopherols on cNOS activity. α-, γ-, and δ-tocopherols did not change cNOS protein expression, but increased phosphorylation of cNOS in platelets. The combination of three tocopherols further increased cNOS activity compared with α-, γ-, and δ-tocopherol alone. This figure is representative of six independently performed experiments.

inition of the biological activity of tocopherols is not in conflict with the platelet inhibitory effect found in this investigation, or smooth muscle cell proliferation inhibition described previously (21). Both these studies show that γ- and δ-tocopherol have almost the same platelet inhibitory activity as α-tocopherol.

Atherosclerosis and thrombosis are associated with many common pathologic features, such as deposition and aggregation of platelets, monocyte/macrophage infiltration, and dysfunctional endothelium. Freedman and associates (22) showed that α-tocopherol may inhibit platelet aggregation by a protein kinase C–dependent mechanism. However, as the other isoforms of tocopherol do not inhibit activation of protein kinase C (21), this may not be a major mechanism of the effect of tocopherols on platelet aggregation. In recent studies (13,14), we demonstrated that both α-tocopherol and γ-tocopherol–rich preparations significantly inhibited lipid peroxidation and platelet aggregation and delayed thrombus formation in the rat, and the γ-tocopherol–rich preparation was more potent than α-tocopherol preparation. We found that both the α-tocopherol and γ-tocopherol–rich preparations also affect the stability of arterial thrombus. This study provides further evidence that the three isoforms of tocopherol individually and similarly inhibit human platelet aggregation. Importantly, the combination of three isoforms of tocopherol in its natural form (15) synergistically inhibits ADP-induced human platelet aggregation.

Experimental evidence indicates that free radical–mediated lipid peroxidation could initiate or promote atherogenesis by directly damaging endothelial cells, and by enhancing the adhesion and activation of neutrophils and platelets (23–25). Holvoet and coworkers (26) showed that oxidation of lipids and lipoproteins leads to atherothrombosis. Alterations in antioxidant levels have been described in patients with coronary heart disease and diabetes mellitus (27,28). Muller and Sorrell (29) showed that lipid peroxidation in platelets enhances activation of platelets. Vitamin E may play a protective role as an antioxidant and/or membrane-stabilizing agent in platelets. Decreased vitamin E levels in platelets are associated with increased aggregation. We observed earlier that both α-tocopherol and γ-tocopherol–rich preparations significantly reduce superoxide anion generation and lipid peroxidation product MDA in rats in vivo (13,14). This study extends these observations in that the three different isoforms of tocopherol exert a similar inhibitory effect on lipid peroxidation in human platelets. The combination of the three isoforms of tocopherol had a more powerful inhibitory effect than individual isoforms.

Previous studies from our laboratory (30) identified a calcium-dependent endothelial-type cNOS isoform in human platelets. The classic inhibitors of cNOS significantly decrease L-arginine (the substrate of NOS) uptake and NOS activity in the platelets. It has been suggested that NO synthesis in platelets may be a regulatory factor in platelet activation. Freedman and associates (31) recently showed that impaired platelet NO production predicts acute coronary syndromes. Other recent studies (13,14) show that both α-tocopherol and γ-tocopherol–rich preparations increase cNOS activity and NO generation in rat aortas. In this study, we found that α-, γ-, and δ-tocopherol each sig-

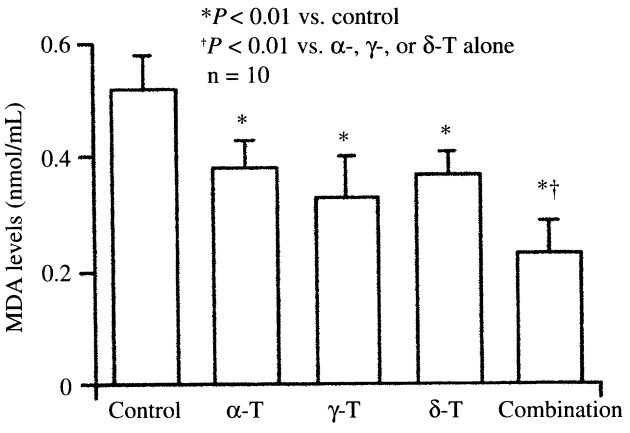

Fig. 5. The effect of tocopherols on lipid peroxidation product MDA. Incubation of PRP with α-, γ-, and δ-tocopherols decreased MDA levels compared with control group ($P < 0.05$). The combination of three tocopherols further decreased MDA levels compared with α-, γ-, or δ-tocopherol alone ($P < 0.05$). Data are mean ±SD from 10 experiments.

nificantly increase NO release. The combination of the three isoforms of tocopherols further increases NO release, which may be a basis for the effect of tocopherols on platelet aggregation.

To determine the basis for the increased NO release, we measured cNOS protein expression and its activity in platelets. Constitutive nitric oxide synthase protein expression was unaffected by different isoforms of tocopherols, as would be expected in anucleated platelets. However, its activity, determined by measurement of its phosphorylation, was significantly enhanced by each isoform of tocopherol. The combination of three isoforms also did not affect cNOS protein expression, but markedly increased cNOS phosphorylation beyond the effect of each isoform.

We suggest that the effect of the combination of tocopherols may be particularly important since natural sources of antioxidants, such as vegetable oils, nuts and grains, contain multiple isoforms of tocopherol. Use of α-tocopherol alone, found in most commercial preparations of vitamin E may not be adequate to combat oxidant stress in vascular disease states. An in vitro study (32) has even suggested that high concentrations of α-tocopherol may become pro-oxidant. The harmful effect of α-tocopherol may be counteracted by γ-tocopherol (9,10). In accordance with the concept that the combination of tocopherols as found in nature may be beneficial, use of fruits and vegetables has been found to be more beneficial than α-tocopherol alone in the prevention of CAD-associated morbidity (9). Recently, a large trial (33) reported that intake of α-tocopherol alone does not provide a survival benefit. The AHA Scientific Council on Nutrition has taken the stand that dietary supplementation with natural sources of vitamin E as in food may be helpful in the primary prevention of CAD (10).

In summary, this study shows that α-, γ-, and δ-tocopherol have similar inhibitory effect on human platelet aggregation and lipid peroxidation. These effects are associated with an increase in platelet cNOS activity. The combination of three tocopherols, as found in nature, has a more powerful effect on these parameters. Future clinical trials should utilize the combination of these three isoforms of tocopherol.

References

1. Diaz MN, Frei B, Vita JA, et al. Antioxidants and atherosclerotic heart disease. N Engl J Med 337:408–416, 1997
2. Keaney JF Jr, Guo Y, Cunningham D, et al. Vascular incorporation of alpha-tocopherol prevents endothelial dysfunction due to oxidized LDL by inhibiting protein kinase C stimulation. J Clin Invest 98:386–394, 1996
3. Keaney JF Jr, Xu A, Cunningham D, et al. Dietary probucol preserves endothelial function in cholesterol-fed rabbits by limiting vascular oxidative stress and superoxide generation. J Clin Invest 95:2520–2529, 1995
4. Fuster V, Badimon L, Badimon JJ, et al. The pathogenesis of coronary artery disease and the acute coronary syndromes. N EngI J Med 326:310–318, 1992
5. Conti CR, Mehta JL. Acute myocardial ischemia: Role of atherosclerosis, in situ thrombosis, platelet activation, coronary vasospasm, and altered arachidonic acid metabolism. Circulation 75 (suppl V):V84–V95, 1987
6. Ikeda H, Koga Y, Oda T, et al. Free oxygen radicals contribute to platelet aggregation and cyclic flow variations in stenosed and endothelium-injured canine coronary arteries. J Am Coll Cardiol 24:1749–1756, 1994
7. Jourdan A, Aguejouf O, Imbault P, et al. Experimental thrombosis model induced by free radicals. Application to aspirin and other different substances. Thromb Res 79: 109–123, 1995
8. Mehta J, Li DY, Mehta JL. Vitamin C and E prolong time to arterial thrombosis in rats. J Nutr 129:109–112, 1999
9. Kushi LH, Folsom AR, Priineas RJ, et al. Dietary antioxidant vitamins and death from cardiovascular disease in post-menopausal women. N Engl J Med 334:1156–1162, 1996
10. Tribble DL. Antioxidant consumption and risk of coronary heart disease: Emphasis on vitamin C, vitamin E, and b-carotene. A statement for healthcare professionals from the American Heart Association. Circulation 99:591–595, 1999
11. Wolf T. Gamma-tocopherol: An efficient protector of lipids against nitric oxide-initiated peroxidative damage. Nutr Rev 55:376–378, 1997
12. Christen S, Woodall AA, Siiigenaga MK, et al. γ-Tocopherol traps mutagenic electrophiles such as NOx and complements α-tocopherol: Physiological implications. Proc Natl Acad Sci USA 94:3217–3222, 1997
13. Saldeen T, Li DY, Mehta JL. Differential effects of α- and γ-tocopherol on low-density lipoprotein oxidation, superoxide activity, platelet aggregation and arterial thrombogenesis. J Am Coll Cardiol 34:1208–1215, 1999
14. Li DY, Saldeen T, Romeo F, et al. Relative effects of α- and γ-tocopherol on LDL oxidation and SOD and NOS activity and protein expression in rats. J Cardiovasc Pharmacol Ther 4:1–8, 1999
15. Baurnfeind T. Tocopherols in foods. In Machlin LT (ed). Vitamin E: A Comprehensive Treatise. Marcel Decker, New York, 1980
16. Chen LY, Haught WH, Yang BC, et al. Preservation of endogenous antioxidant activity and inhibition of lipid peroxidation as common mechanisms of antiatherosclerotic effects of vitamin E, lovastatin and amlodipine. J Am Coll Cardiol 30:569–575, 1997
17. Li DY, Yang BC, Mehta JL. Ox-LDL induces apoptosis in human coronary artery endothelial cells: Role of PKC, PTK, bcl-2, and Fas. Am J Physiol 275:H568–H576, 1998
18. Chen LY, Mehta P, Mehta JL. Oxidized LDL decreases L-arginine uptake and nitric oxide synthase protein expression in human platelets: Relevance of the effect of oxidized LDL on platelet function. Circulation 93:1740–1746, 1996

19. Li DY, Tomson K, Yang BC, et al. 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 41:109–115, 1999
20. The Food and Nutrition Board of the National Research Council: National Research Council (NRC) Recommended Dietary Allowances. 10th edition. National Academy Press, Washington DC, 1989
21. Chatelain E, Boscoboinik DO, Bartoll GM, et al. Inhibition of smooth muscle cell proliferation and protein kinase C activity by tocopherols and tocotrienols. Biochem Biophys Acta 1176:83–89, 1993
22. Freedman JE, Farhat JH, Loscalzo J, et al. Alpha-tocopherol inhibits aggregation of human platelets by a protein kinase C-dependent mechanism. Circulation 10: 2434–2440, 1996
23. Hennig B, Chow CK. Lipid peroxidation and endothelial cell injury: Implications in atherosclerosis. Free Radical Biol Med 4:99–106, 1988
24. Poch B, Gansauge F, Rau B, et al. The role of polymorphonuclear leukocytes and oxygen-derived free radicals in experimental acute pancreatitis: Mediators of local destruction and activators of inflammation. FEBS Lett 461:268–272, 1999
25. Lawson DL, Mehta JL, Nichols WW, et al. Superoxide radical-mediated endothelial injury and vasoconstriction of rat thoracic aortic rings. J Lab Clin Med 115:541–548, 1990
26. Holvoet P. Oxidative modification of low-density lipoproteins in atherothrombosis. Acta Cardiol 53:253–260, 1998
27. Buczynski A, Wachowicz B, Kedziora-Kornatowska K, et al. Changes in antioxidant enzymes activities, aggregability and malonyldialdehyde concentration in blood platelets from patients with coronary heart disease. Atherosclerosis 100:223–228, 1993
28. Dmoszynska A, Walter-Croneck A, Ledwozyw A. Lipid peroxidation products and changes in phospholipid composition induced by indobufen in diabetic platelets. Thromb Res 79:483–490, 1995
29. Muller M, Sorrell TC. Oxidative stress and the mobilization of arachidonic acid in stimulated human platelets: Role of hydroxyl radical. Prostaglandins 54:493–509, 1997
30. Chen LY, Mehta JL. Further evidence of the presence of constitutive and inducible nitric oxide synthase isoforms in human platelets. J Cardiovasc Pharmacol 27:154–158, 1996
31. Freedman JE, Ting B, Hankin B, et al. Impaired platelet production of nitric oxide predicts presence of acute coronary syndromes. Circulation 98:1481–1486, 1998
32. Bowry VW, Ingold KU, Stocker R. Vitamin E in human low-density lipoprotein. When and how this anti-oxidant becomes a pro-oxidant? Biochem J 288:341–344, 1992
33. The Heart Outcomes Prevention Evaluation Study Investigators. Vitamin E supplementation and cardiovascular events in high risk patients. N Engl J Med 342: 154–160, 2000