Vitamin E Inhibits Collagen-Induced Platelet Activation by Blunting Hydrogen Peroxide

Pasquale Pignatelli, Fabio M. Pulcinelli, Luisa Lenti, Pier Paolo Gazzaniga, Francesco Violi

Abstract—In this study, we investigated whether vitamin E at concentrations achievable in blood after supplementation inhibits platelet function in humans. Gel-filtered platelets were incubated 30 minutes with scalar concentrations (50 to 250 mmol/L) of vitamin E and then stimulated with collagen. Compared with controls, vitamin E inhibited collagen-induced platelet aggregation and thromboxane A2 formation in a dose-dependent manner. Furthermore, vitamin E inhibited, in a dose-dependent manner, Ca2+ mobilization and formation of inositol 1,4,5-triphosphate. Because it was previously shown that hydrogen peroxide formation mediates arachidonic acid metabolism and phospholipase C activation in collagen-induced platelet activation, we investigated whether vitamin E was able to blunt hydrogen peroxide. In experiments performed in unstimulated platelets supplemented with hydrogen peroxide and in collagen-stimulated platelets, vitamin E was able to blunt hydrogen peroxide. In 6 healthy subjects given vitamin E for 2 weeks (600 mg/d), we found a significant decrease of collagen-induced H2 O2 formation, platelet aggregation, and calcium mobilization. This study demonstrated in vitro and ex vivo that vitamin E inhibits collagen-induced platelet activation by blunting hydrogen peroxide formation. (Arterioscler Thromb Vasc Biol. 1999;19:2542-2547.)

Key Words: antioxidants ■ platelet aggregation ■ arachidonic acid metabolism ■ phospholipase C enzyme ■ thromboxane A2

Use of antioxidants to prevent coronary heart disease is based on the assumption that atherosclerosis progression is determined by oxidative modification of LDL, which is then taken up by macrophages of atherosclerotic plaque.1 Among antioxidants, vitamin E has been widely investigated to assess its clinical effectiveness. In healthy subjects, dietary vitamin E consumption has been associated with fewer cardiovascular events.2,3 In patients with angiographically proven coronary heart disease, 400 to 800 mg/d vitamin E significantly reduced the rate of nonfatal myocardial infarction over 2 years of follow-up.4 These data have not been confirmed by other studies, such as that of Rapola et al;5 however, in that study, daily administration of vitamin E was only 50 mg. Despite these contradictory results, additional studies have been conducted to assess whether vitamin E has biological properties that could prevent progression of atherosclerosis.

Several in vitro and in vivo studies have analyzed whether vitamin E inhibits platelet function. Recent studies demonstrated that vitamin E supplementation in healthy subjects or patients with hypercholesterolemia diminishes platelet function, as assessed by ex vivo platelet aggregation and urinary excretion of 11-dehydro-thromboxane B2, a marker of in vivo platelet activation.6,7 Inhibition of platelet function was dependent on the daily dose of vitamin E, indicating that plasma and platelet vitamin E concentrations are crucial for the inhibition of platelet function. These data seem to contradict those of in vitro studies, in which higher concentrations of vitamin E (0.5 to 1 mmol/L) than observed in plasma after supplementation were necessary to inhibit human platelet activity.8,9 To analyze this discrepancy, we performed an in vitro study using collagen as the platelet agonist. Collagen is an important platelet agonist thought to be involved in the early stages of platelet activation during both hemostasis and thrombosis. We recently demonstrated that collagen-induced platelet aggregation is associated with a burst of hydrogen peroxide, an oxidant species that contributes to activation of platelets.10 Because vitamin E is an antioxidant, we speculated that collagen-induced platelet aggregation may represent an interesting model to further analyze the relationship between vitamin E and platelet function. We found that a concentration of vitamin E very close to that found in human plasma after supplementation is able to inhibit collagen-induced platelet activation by blunting hydrogen peroxide formation.

Methods

Materials

3Pi was from Amersham. Fura 2-AM and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were from Molecular Probes, and Sepharose 2B was from Pharmacia. Collagen, type 1, was from Semmelweis.

High-performance liquid chromatography (HPLC) columns (Partisil

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10 SAX) were from Whatman. BSA, HEPES, acetylcholine chloride, α-tocopherol (vitamin E), fibrinogen, inorganic pyrophosphate, acid/citrate/dextrose, digitonin, EGTA, EDTA, Tris, perchloric acid, formaldehyde, indomethacin, ammonium formate, maleic acid, creatine phosphate, and creatine phosphokinase were from Sigma Chemical Co.

Platelet Preparation

Human blood was obtained from drug-free healthy volunteers and anticoagulated with acid/citrate/dextrose.11 Platelet-rich plasma, obtained by centrifugation (15 minutes at 1800 g), was recentrifuged (20 minutes at 8000 g) to concentrate the platelets, and the pellet was resuspended in a 0.5 vol of autologous platelet-poor plasma. Platelet suspensions were incubated for 1 hour at 37°C with Fura 2-AM (3 mmol/L of cell suspension), DCFH-DA (40 mmol/L), or 32Pi (2 mCi/mL).

Platelets were separated from plasma proteins and from excess Fura 2-AM and 32Pi by gel filtration on Sepharose 2B using Ca2+-free Tyrode’s buffer containing 0.2% BSA, 5 mmol/L glucose, and 10 mmol/L HEPES, pH 7.35. After gel filtration, the cell suspension (gel-filtered platelets [GFPs]) was adjusted to a final concentration of 2×107 cells/mL.

Because the addition of methanol to GFP suspensions in concentrations lower than 0.5% did not induce any change in GFP response to collagen in preliminary experiments, this ratio was always used to obtain final concentrations of vitamin E of 50 to 250 mmol/L. Vitamin E was added to GFP suspensions under continuous stirring for 30 minutes at 37°C before GFP stimulation with collagen (2 to 12 μg/mL). According to results of our previous study,10 the collagen concentrations that gave reproducible values varied on the basis of the test used, ie, a higher concentration of collagen was necessary to induce inositol 1,4,5-trisphosphate (IP3) formation compared with the test used. GFPs were incubated with a dual-channel (3H-32P) HPLC radioactivity detector (Flo-One SFM 25 fluorometer, Kontron) set at 340 nm excitation and 510 nm emission. To convert fluorescence measurements into Ca2+ concentrations, minimum fluorescence was determined after addition of digitonin (50 mmol/L) in the presence of EGTA (2 mmol/L) and Tris base (20 mmol/L); maximum fluorescence was measured by addition of excess CaCl2 (10 mmol/L). The calcium concentration was calculated using these values and a Kd of 224 mmol/L, according to the method of Grynkiewicz et al,13 after correction for extracellular dye.

Phospholipase C Activation

Because activation of phospholipase C (PLC) produces IP3 from phosphatidylinositol-4,5-bis-phosphate, and because IP3 is converted within 30 to 60 seconds into inositol 1,3,4,5-tetrakisphosphate, which is rapidly degraded into the more stable inositol 1,3,4,5-tetrakisphosphate,14 we studied inositol 1,3,4,5-tetrakisphosphate production 1 minute after platelet stimulation with 12 μg/mL of collagen, which was the lowest collagen concentration able to induce a reproducible response. Collagen-stimulated activation of the [3H]-labeled platelets, resuspended in phosphate-free Tyrode’s buffer, was stopped by means of perchloric acid (0.44 N). The neutralized platelet extracts (1×107 cells/mL) were treated overnight with Zn2+-pyrophosphatase (20 U/mL) in the presence of Tris-maleic acid (1.5 mmol/L; pH 7.35) and then passed on an HPLC column eluted with a 50-minute linear gradient that used water as the first buffer and ammonium formate (15 mmol/L; pH 3.75) as the final buffer. Inositol peaks were detected with a dual-channel (H-32P) HPLC radioactivity detector (Flo-One A100 Radiomatic, Camberra Co) that used [3H]-inositol 1,3,4-trisphosphate as the pure standard.14

Ex Vivo Study

In 6 healthy volunteers (3 men and 3 women; age, 30 to 48 years), collagen-induced platelet H2O2 formation, platelet aggregation, calcium mobilization, and vitamin E plasma levels were measured before and after 2 weeks of oral vitamin E supplementation (600 mg/d). Collagen-induced platelet activation was analyzed by using the methods described above; all experiments were performed within 10 minutes after gel filtration. The plasma concentration of vitamin E was measured by HPLC according to the method of Bieri et al.15

A flow rate of 2.0 mL/min was used on an octadecilcica C18 (ODS) 5 μm with a LC/233 diode array detector (Restek Corp) set at 0.02 to 0.1 attenuation.

Statistical Analysis

Data are reported as mean±SEM. Comparisons between variables were analyzed by Student’s t test for paired and unpaired data. Significance was set at P<0.05.

Results

In Vitro Study

Flow Cytometric Analysis

Platelet-rich plasma was recentrifuged (800 g for 20 minutes) to concentrate the platelets, and the pellet was resuspended in PBS. DCFH-DA (8 mL/mL; 5-mmol/L concentration) was added, and after 15 minutes of incubation, the platelet suspension was washed twice and resuspended in Tyrode’s buffer at a final concentration of 2×107 cells/mL. The platelet preparation was activated with collagen, added with or without vitamin E, and the reaction was stopped after 1 minute with EGTA (2 mmol/L).

All samples were analyzed on a Coulter Epics flow cyometer equipped with an argon laser (480 nm emission). The instrument was set up to measure logarithmic forward light scatter, a measure of particle size; logarithmic 90° light scatter, a measure of cell granularity; and green (dichlorofluorescin [DCF]) fluorescence (at 510 to 550 nm). Fluorescent parameters (expressed in arbitrary units [au]) were collected using 3-decade logarithmic amplification.

Platelet Aggregation

In vitro platelet aggregation was evaluated according to the method of Born12 in a 4-sample Aggrecorder II (Menarini) at 37°C using siliconized glass cuvetts under continuous stirring at 1000 rpm. Fibrinogen (1 mg/mL) was added before the agonist.

Production of Thromboxane A2

Platelet activation (see above) was stopped after 3 minutes with indomethacin (14 mmol/L). Thromboxane A2 production was determined by using thromboxane B2 ELISA kits (Boehringer Mannheim GmbH).

Platelet Cytosolic Ca2+ Concentrations

Calcium concentrations were measured by using the fluorescent indicator dye Fura-2. Changes in fluorescence were monitored with a SFM 25 fluorometer (Kontron) set at 340 nm excitation and 510 nm emission. To convert fluorescence measurements into Ca2+ concentrations, minimum fluorescence was determined after addition of digitonin (50 mmol/L) in the presence of EGTA (2 mmol/L) and Tris base (20 mmol/L); maximum fluorescence was measured by addition of excess CaCl2 (10 mmol/L). The calcium concentration was calculated using these values and a Kd of 224 mmol/L, according to the method of Grynkiewicz et al,13 after correction for extracellular dye.

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Results

In Vitro Study

Flow Cytometric Analysis

The flow cytometric method uses the properties of DCFH-DA,16–18 which rapidly diffuses across cell membranes and is then trapped within the cell by a deacetylation reaction. In the presence of hydrogen peroxide, this compound is oxidized to DCF, which is highly fluorescent.19 Figure 1 shows the DCF green fluorescence distribution of unactivated platelets (31±2.8 au, Figure 1A). The largest increase of DCF fluorescence occurred with 0.1 mmol/L H2O2 (74.1±4.3 au, P<0.01 versus control, Figure 1B). Collagen-induced platelet activation doubled the shift of DCF fluorescence in comparison to unstimulated platelets (65.0±7 au, P<0.02 versus control, Figure 1C). Vitamin E inhibited the shift of DCF fluorescence induced by collagen in a concentration-dependent manner (with 50 μmol/L, DCF fluorescence=43±7 au, P<0.05 versus collagen alone, Figure 1D; with 125 μmol/L, 28.8±3 au, P<0.05 versus collagen alone, Figure 1E; and with 250 μmol/L, 24±3 au, P<0.003 versus collagen alone, Figure 1F). To investigate the mechanism underlying such effects, we analyzed whether vitamin E was able to quench H2O2 in a system containing unactivated platelets. As shown in Figure 2, we found that vitamin E...
quenched up to $1 \times 10^{-4}$ mmol/L H$_2$O$_2$. The fact that collagen induced a fluorescence shift lower than that induced by $1 \times 10^{-3}$ mmol/L H$_2$O$_2$ might suggest that in vitamin E-treated platelets stimulated with collagen, the decrease of fluorescence is due to a direct interaction between vitamin E and H$_2$O$_2$ produced by platelets.

**Platelet Aggregation**

Inhibition of platelet aggregation by vitamin E was closely dependent on incubation time, with $>50\%$ inhibition observed after at least 15 minutes of incubation (Figure 3). More than $80\%$ inhibition was observed after 30 minutes of incubation; therefore, in the following experiments, platelets were incubated with vitamin E for 30 minutes before collagen was added. We found that vitamin E inhibited collagen-induced platelet aggregation depending on the concentration used. A $50\mu$mol/L concentration of vitamin E significantly inhibited platelet aggregation; almost complete inhibition of platelet aggregation was observed with $250\mu$mol/L (Figure 4). No inhibition was observed in adenosine diphosphate, arachidonic acid, U46619, or thrombin-activated platelets (data not shown).

**Platelet Thromboxane A2 Formation**

Collagen-induced thromboxane A2 formation was inhibited in a dose-dependent manner by vitamin E (Figure 5). A 10-$\mu$g/mL concentration of collagen produced 67±7 ng/mL thromboxane A2, which was reduced to 35±3 ng/mL ($P<0.05$), 19±10 ng/mL ($P<0.01$), and 11±3 ng/mL ($P<0.01$) by 50, 125, and 250 $\mu$mol/L vitamin E, respectively.

**Changes in Intracellular Calcium Concentration**

Percentages of change in intracellular calcium concentration induced by collagen in control GFPs and GFPs treated with...
vitamin E (50 to 250 μmol/L) are reported in Figure 6. In samples stimulated with collagen (2 μg/mL), vitamin E inhibited intracellular calcium mobilization in a dose-dependent manner.

**PLC Activation**
Production of 32P-labeled inositol 1,3,4-triphosphate by collagen-stimulated platelets (12 μg/mL), added with and without vitamin E, is shown in Figure 7. Collagen-induced formation of inositol 1,3,4-triphosphate was inhibited by vitamin E in a dose-dependent manner.

**Ex Vivo Study**
In 6 healthy subjects given vitamin E, the plasma concentration of vitamin E rose from 19.0±3.1 to 75.0±8.4 μmol/L (P<0.05). After vitamin E supplementation, we observed a significant decrease of collagen-induced platelet H2O2 formation, platelet aggregation, and calcium mobilization (Table).

**Discussion**
This study showed that a concentration of vitamin E as low as 50 μmol/L is able to inhibit collagen-induced platelet activation by interfering with arachidonic acid metabolism and the PLC pathway. We recently demonstrated that during platelet aggregation by collagen, there is a burst of hydrogen peroxide that amplifies the platelet response to the agonist.10 Thus, hydrogen peroxide stimulated arachidonic acid metabolism, contributing to platelet production of thromboxane A2; in addition, hydrogen peroxide stimulated IP₃ formation and Ca²⁺ mobilization. In this experimental model, vitamin E inhibited platelet thromboxane as well as IP₃ formation and Ca²⁺ mobilization, suggesting that it influences both arachidonic acid metabolism and the PLC pathway. The inhibition of platelet function by vitamin E was due to its interaction with hydrogen peroxide. Thus, in experiments performed by incubating unstimulated platelets with scalar concentrations of hydrogen peroxide, vitamin E blunted hydrogen peroxide depending on the concentration of hydrogen peroxide present in the system; with a concentration of hydrogen peroxide of 1×10⁻⁴ mmol/L, 250 μmol/L vitamin E almost completely prevented the hydrogen peroxide–mediated increase in DCF fluorescence. In collagen-stimulated platelets, vitamin E blunted hydrogen peroxide formation in a dose-

**Figure 4.** Vitamin E inhibited collagen-induced (2 μg/mL) platelet aggregation in a dose-dependent manner. Data are mean±SEM of 5 experiments. *P<0.01; **P<0.005.

**Figure 5.** Vitamin E inhibited thromboxane B2 (TXB₂) production in collagen-stimulated platelets. Platelet thromboxane B2 production in collagen-stimulated (10 μg/mL) platelets in the absence and presence of scalar doses (50 to 250 μmol/L) of vitamin E. Data are mean±SEM of 5 experiments. *P<0.05; **P<0.01.

**Figure 6.** Vitamin E inhibited collagen-induced calcium mobilization. Percentage of change (Δ) of calcium mobilization in collagen-stimulated platelets in the absence and presence of scalar doses (50 to 250 μmol/L) of vitamin E. Data are mean±SEM of 5 experiments. *P<0.05; **P<0.01.

**Figure 7.** 32P-labeled inositol 1,3,4-triphosphate (Ins 1,3,4 P₃) production induced by collagen-stimulated (12 μg/mL) platelets in the absence and presence of scalar doses (50 to 250 μmol/L) of vitamin E. Data are mean±SEM of 5 experiments. *P<0.05; **P<0.01.
Plasma Vitamin E Concentration, Platelets H2O2 Formation (Baseline and After Collagen Stimulus), Platelet Aggregation, and Calcium Mobilization Before and After Vitamin E Supplementation (600 mg/d) in 6 Healthy Volunteers

<table>
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<th>Subject</th>
<th>Vitamin E, μmol/L Before</th>
<th>After</th>
<th>DCFH Oxidation, au Baseline</th>
<th>Collagen Stimulus</th>
<th>Aggregation, maximum % Before</th>
<th>After</th>
<th>Ca2+ Mobilization, Δ% Before</th>
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Mean±SEM: 19.0±3.1 75.0±8.4 32.5±3.3 19.4±4.2 58.5±9.1 15.0±6.4 52.5±7.4 35.2±4.2 307.7±66.3 136.7±76.5

Collagen concentrations were the same for in vitro experiments. All differences before and after vitamin E supplementation are <0.05.

dependent manner. Inhibition was evident with concentrations of vitamin E as low as 50 μmol/L, because in collagen-stimulated platelets, the concentration of hydrogen peroxide is much lower than 1×10^{-3} μmol/L. Together, these findings indicate that vitamin E inhibits platelet function through its antioxidant properties, in particular by blocking the capacity of hydrogen peroxide to stimulate platelet arachidonic acid metabolism and the PLC pathway.

Our results differ from those of previous studies showing that concentrations of vitamin E much higher than those found in human plasma after supplementation are necessary to reduce in vitro platelet function. Recently, Freedman et al. reported that 0.5 μmol/L vitamin E inhibits platelet aggregation by interfering with the protein kinase C pathway, with no evidence of platelet inhibition using lower concentrations. In this study, however, the relationship between vitamin E and collagen-induced platelet aggregation was not investigated.

In the same study, it was also demonstrated that incorporation of vitamin E into platelet membrane was time-dependent; incorporation was crucial to obtaining evident platelet inhibition. Even if we did not measure vitamin E incorporation into platelets, our finding is in agreement with the demonstration that vitamin E inhibits platelet aggregation depending on its incubation time. In this study, we observed the maximum effect on platelet inhibition by incubating platelets with vitamin E for 30 minutes.

Our finding may be of clinical relevance because it provides experimental support for 2 recent studies showing that vitamin E supplementation in humans decreases ex vivo and in vivo platelet activation. Both of these studies indicate a close relationship between plasma vitamin E levels and platelet activation (ie, higher plasma levels of vitamin E and lower platelet function). In particular, a plasma vitamin E concentration of about 50 μmol/L was associated with ex vivo and in vivo platelet inhibition, which is in accord with our data indicating that 50 μmol/L vitamin E inhibits in vitro platelet activation. This suggestion is supported by results of our ex vivo study, which showed that vitamin E significantly inhibits collagen-induced platelet activation; interestingly, after supplementation, the plasma concentration of vitamin E was very close to that shown in vitro to inhibit collagen-induced platelet activation. However, there is a discrepancy between the results of the in vitro and ex vivo studies that requires more investigation. The ex vivo study showed complete suppression of platelet H2O2 formation, but this was observed in vitro only with the addition of 250 μmol/L. However, this difference could be due to the different incubation times that were used in the in vitro and ex vivo studies.

In conclusion, these in vitro and ex vivo studies showed that vitamin E inhibits collagen-induced platelet activation. This effect is due to its interaction with hydrogen peroxide, an important mediator of collagen-induced platelet activation. This finding gives new insight into the mechanism of action of vitamin E and provides experimental support for its possible use as an antiplatelet agent.

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References


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