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Regular Article

Vitamin E inhibition on platelet procoagulant activity: Involvement of aminophospholipid translocase activity

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ABSTRACT

Background: Activated platelets provide an important procoagulant surface, because exposed negatively charged phosphatidylserine (PS) is an important cofactor of the coagulation cascade. Aminophospholipid translocase (APLT) can transport PS from the outer to the inner membrane leaflet. Although vitamin E has been investigated for its anti-aggregating effect on platelets, its effect on platelet procoagulant activity has not been reported.

Methods: Phorbol 12-myristate 13-acetate (PMA), a well-known PKC activator, and thrombin were used to induce PS exposure on platelet surface. The expression of PS was measured by annexin A5 binding with flow cytometry. Platelet procoagulant activity was measured by a prothrombinase assay. APLT activity was measured by flow cytometry by determining the percent of 1-palmitoyl-2-[6-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino] caproyl]-sn-glycero-3-phosphatidylserine (NBD-PS) translocated from the outer to the inner membrane leaflet. Inhibition effects of vitamin E on platelet aggregation were simultaneously measured by a Multiplate aggregometer, a Chrono-log aggregometer, and a PFA-100 system.

Results: Vitamin E significantly attenuated PMA-induced conformational change of glycoprotein IIb/IIIa and P-selectin expression. Vitamin E significantly inhibited PMA and thrombin-induced PS externalization and reduced prothrombinase activity on platelet surfaces both *in vitro* and *ex vivo*. APLT activity was increased by vitamin E in a dose-dependent manner, indicating that reduced procoagulant activity may be attributed, at least in part, to this increased APLT activity. Vitamin E inhibited platelet aggregation induced by combined chemokine SDF-1 and low-dose ADP as well as by usual doses of ADP or collagen when measured by the Multiplate and Chrono-log aggregometers but not when measured by PFA-100.

Conclusions: These *in vitro* and *ex vivo* results showed that vitamin E inhibited platelet PS exposure and procoagulant activity partly by increasing APLT activity. These actions of vitamin E on platelet function provide new insights into the anticoagulation properties of vitamin E.

Introduction

Vitamin E is an antioxidant that has been proven to exert a beneficial effect on cardiovascular disease. Although the effect of vitamin E has generally been attributed to its antioxidant activity by virtue of its ability to inhibit LDL oxidation[1], it is also known to inhibit platelet aggregation and adhesion elicited by phorbol 12-myristate 13-acetate (PMA) by interfering with protein kinase C (PKC) activity[2].

The lipid bilayer membrane of resting platelets is composed of cholinephospholipids in the outer leaflet and negatively charged phosphatidylserine (PS) and phosphatidylethanolamine (PE) in the inner leaflet[3]. Aminophospholipid translocase (APLT) helps maintain phospholipid asymmetry by transporting the PS from the outer to the inner membrane leaflet. Scramblase is also responsible for the rapid transfer of PS and PE from the inner too the outer membrane leaflet. Activated platelets provides an important procoagulant surface, because exposed negatively charged PS is an important cofactor of the coagulation cascade, which includes activation of factor X and prothrombin. Platelet procoagulant activity is largely determined by surface PS exposure.

Annexin A5 is a positively charged glycoprotein that binds to phospholipids and has high affinity in the presence of micromolar concentration of calcium[4]. Annexin A5 is used to detect PS exposure on the plasma membrane of nucleated cells.

Meanwhile, PS externalization during apoptosis is preceded by PS oxidation. Lipid antioxidants are capable of blocking PS oxidation, resulting in inhibition of PS externalization[5]. Etoposide, a lipid antioxidant, was also reported to inhibit PS externalization of monocytic cells by preventing PS oxidation[6]. Because vitamin E is an antioxidant, it is hypothesized that vitamin E may inhibit

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platelet procoagulant activity by blunting PS externalization. Accordingly, we investigated whether vitamin E (α-tocopherol, the main vitamin E isomer) would inhibit PS exposure and the consequent change of procoagulant activity on the surface of platelets. Because vitamin E is particularly effective at inhibiting platelet function to PKC-dependent agonist[2], we used PMA, a well-known PKC activator, to induce PS exposure on the platelet surface. To investigate the inhibitory mechanism of PS exposure, APLT activity was measured in vitamin E-treated platelets. Furthermore, we analyzed the anti-aggregating effect of vitamin E with a rapid Platelet Function Analyzer (PFA)-100^{TM} (Dade-Behring, Liederbach, Germany) and conventional platelet aggregometers of whole blood and platelet-rich plasma (PRP). Finally, to explore the effect of vitamin E on platelet aggregation in inflammatory status, chemokine stromal-derived factor (SDF)-1 combined with ADP was used as a platelet agonist.

Methods

Subjects

Peripheral blood from healthy volunteers (n = 21, Male 10, Female 11, mean age 29.0 ± 5.4 years) was collected using sodium citrate tubes (Becton Dickinson, San Jose, CA, USA) after informed consent was obtained. All volunteers denied taking medication affecting platelet function for 14 days preceding the study. For *ex vivo* experiments, three healthy volunteers received oral vitamin E (d-α-tocopherol, 670 mg/d, Yuhan Corporation, Seoul, Korea) supplementation for 3 weeks. Before and after the oral supplementation, the peripheral blood was taken for assessment of PS exposure and APLT activity in platelets.

Flow cytometric analysis of platelet surface receptors and PS exposure

PRP was made after whole blood was centrifuged at 800 × *g* for 10 minutes, and the resulting platelets was re-suspended at a concentration of 1×10^9/mL in phosphate buffered saline (PBS) containing 1 mM Ca^{++}. These platelet suspensions were pre-incubated with vitamin E (Sigma Aldrich, St. Louis, MO) or vehicle (DMSO) for 10 min and stimulated with a PKC activator, PMA (Sigma Aldrich) or thrombin (Sigma Aldrich) at 37 °C for 10 min. A PKC inhibitor (GÖ6976, Sigma Aldrich) was used in a separate experiment. The platelets were stained with fluorescein isothiocyanate (FITC)-conjugated PAC1, allophycocyanin (APC)-conjugated anti-P-selectin, or phycoerythrin (PE)- or APC-conjugated annexin A5 (BD Biosciences). The suspensions were analyzed with a FACSCalibur cytofluorometer (BD Biosciences). Data were collected from at least 30×10^3 viable cells and analyzed using WinMDI 2.7 software.

Platelet prothrombinase assay

Platelet procoagulant activity was assayed as described by Thiagarajan *et al.*[7] with some modifications. Platelets were suspended in HEPES buffer containing 3 mmol/l $CaCl_2$ and 0.5 mg/ml bovine serum albumin (BSA, Difco Laboratories, Detroit, MI, USA) and were incubated with 0.1 nmol/l factor Xa and 2 nmol/l factor Va (HYPHEN BioMed, Neuville-sur-Oise, France) for 1 min at 37 °C. Thrombin formation was initiated by addition of 1 μmol/l prothrombin (HYPHEN BioMed) and arrested after 2 min by addition of 5 mmol/l EDTA. Thrombin was measured using a thrombin specific chromogenic substrate (HYPHEN BioMed). A commercially prepared lysed platelet concentrate (HYPHEN BioMed) was used as a calibrator, and the results are expressed as nM PS equivalent.

Measurement of the APLT activity

APLT activity was measured using flow cytometry by translocation of fluorescently labeled phospholipid from the outer to the inner membrane leaflet, as described by Chang *et al.*[8]. Briefly, platelets were re-suspended in Tyrode's-HEPES buffer (136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.4). After incubation with vitamin E and PMA or thrombin stimulation, 5 μg/ml 1-palmitoyl-2-[6-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino] caproyl]-sn-glycero-3-phosphatid- ylserine (NBD-PS, Avanti Polar Lipids, Alabaster, AL, USA) was added. Five minutes later, two aliquots were withdrawn and re-suspended with Tyrode's buffer with or without 1% BSA (Difco Laboratories) for 5 min. BSA extracts only surface-associated fluorescence. The suspensions were analyzed by a FACS Calibur cytofluorometer. Results are expressed as % NBD-PS translocated to the inner leaflet by comparing non-extractable fluorescence to total platelet-associated fluorescence.

Measurement of platelet aggregation

Whole blood aggregation was measured with an impedance aggregometer (Multiplate® analyzer, Dynabyte Medical, Munich). After incubation of blood (300 μL) with vitamin E for 5 minutes at 37 °C, platelet agonists (6.45 μM ADP or 1.0 μg/mL collagen, Sigma Aldrich) or SDF-1 (R&D Systems, Minneapolis, MN, USA) were added and the reaction was monitored for 8 minutes. The Chrono-log 540 aggregometer (Chrono-log Corp., Haverton, PA, USA) was used to measure PRP aggregation with the same conditions as those of whole blood. Another set of whole blood aggregation was measured for closure times with collagen/ADP and collagen/epinephrine cartridges of the PFA-100 analyzer.

Statistical analysis

Results are reported as means ± standard error (SE) from 4 to 8 experiments performed on different samples. Statistical comparisons of samples were conducted by Student's t-test using SPSS version 17.0 (SPSS Co., Chicago, IL, USA). *P* values of <0.05 were considered statistically significant.

Results

Effects of vitamin E on the activated status of platelet surface receptors

To demonstrate the inhibitory effect of vitamin E on the activation status of platelet surface receptors, platelet suspensions were pre-incubated with vitamin E or vehicle (0.5% DMSO) for 10 min. Final vitamin E concentrations ranged from 0.05 mM to 0.1 mM, which are close to blood concentration achievable after vitamin E supplementation[9]. After incubation, the platelets were stimulated with 50 nM PMA, a PKC activator. The conformational change of the glycoprotein IIb/IIIa complex of platelet activation was then measured using monoclonal antibody PAC1, which recognizes an epitope on the glycoprotein IIb/IIIa complex of activated platelets. PMA (50 nM) markedly increased the conformational change of the glycoprotein IIb/IIIa complex in mean fluorescence intensity (MFI). Pre-incubation of vitamin E (0.05 and 0.10 mM) significantly inhibited the PMA-induced conformational change of glycoprotein IIb/IIIa (Fig. 1A, B). Likewise, PMA significantly induced P-selectin expression on platelet surfaces, and pre-incubation with vitamin E attenuated PMA-induced P-selectin expression (Fig. 1C, D). The concentration of 50 nM PMA did not significantly increase annexin A5 binding to the platelet surface, and the inhibitory activity of vitamin E on annexin A5 binding was not significant at concentrations of 0.05–0.1 mM vitamin E (data not shown).