Inhibition of Platelet Adherence to Mononuclear Cells by \(\alpha\)-Tocopherol

Role of P-Selectin

Toyoaki Murohara, MD, PhD; Hisao Ikeda, MD, PhD; Yoritaka Otsuka, MD; Mika Aoki, BS; Nobuya Haramaki, MD, PhD; Atsushi Katoh, MD, PhD; Yoshinori Takajo, MD, PhD; Tsutomu Imaizumi, MD, PhD

Background—Platelet-leukocyte interaction is an early important event for thrombogenesis, and this process is mainly mediated by P-selectin on platelets. Although \(\alpha\)-tocopherol has been shown to inhibit thrombotic disorders, the effect of \(\alpha\)-tocopherol on platelet P-selectin expression and platelet-leukocyte interaction is little known.

Methods and Results—We examined whether \(\alpha\)-tocopherol inhibited human platelet P-selectin expression and platelet-leukocyte interaction. \(\alpha\)-Tocopherol (50 to 500 \(\mu\)g/mL) inhibited thrombin-induced or phorbol 12-myristate 13-acetate (PMA)-induced P-selectin expression on platelets. \(\alpha\)-Tocopherol suppressed platelet–mononuclear cell (MNC) interaction, platelet aggregation, and platelet protein kinase C (PKC) activity stimulated with either PMA (100 nmol/L) or thrombin. Inhibitory actions of \(\alpha\)-tocopherol against the platelet functions were mimicked by staurosporine, a selective PKC inhibitor. After oral supplementation of \(\alpha\)-tocopherol (300 mg/d for 3 weeks) in healthy subjects, thrombin-mediated or PMA-mediated P-selectin expression, platelet-MNC interaction, and platelet aggregation ex vivo were suppressed.

Conclusions—\(\alpha\)-Tocopherol inhibited P-selectin expression on human platelets and thereby attenuated platelet-MNC interactions, which were mediated at least in part by the inhibition of intraplatelet PKC activity. These actions of \(\alpha\)-tocopherol on platelet functions provide new insights into the antithromboatherogenic properties of \(\alpha\)-tocopherol. (Circulation. 2004;110:141-148.)

Key Words: thrombus \(\bullet\) platelets \(\bullet\) leukocytes \(\bullet\) antioxidants \(\bullet\) hemorrhage

Platelet–mononuclear cell (MNC) interaction contributes to early process of thrombus formation.\(^1\)\(^-\)\(^3\) In fact, MNCs accumulate around platelet-rich thrombi at sites of hemorrhage and/or ruptured plaques.\(^3\) Platelet-MNC interaction is mediated by various cell adhesion molecules, and P-selectin expressed on activated platelets plays a major role.\(^4\)\(^-\)\(^8\) We previously showed that platelet-MNC interaction by P-selectin contributed to thrombus formation in animal models of acute coronary syndrome.\(^9\)\(^-\)\(^11\)

\(\alpha\)-Tocopherol, a lipophilic antioxidant vitamin,\(^12\) has been shown to inhibit atherothrombotic disorders.\(^13\)\(^-\)\(^16\) Although the effects of \(\alpha\)-tocopherol on vascular endothelial function have been extensively investigated, the effect of \(\alpha\)-tocopherol on human platelet P-selectin expression and platelet-MNC interaction is little known. Accordingly, we examined whether \(\alpha\)-tocopherol would inhibit P-selectin expression on human platelets and whether \(\alpha\)-tocopherol inhibited platelet-MNC interaction that mainly depends on P-selectin expressed on activated platelets.\(^4\)\(^-\)\(^8\) We also examined whether oral supplementation of \(\alpha\)-tocopherol in healthy human subjects would suppress platelet P-selectin expression and platelet-MNC interaction ex vivo.

Methods

In Vitro Experiments

Isolation of Human Platelets and Mononuclear Cells

Peripheral blood (30 mL) was drawn from 37 healthy volunteers and anticoagulated with sodium citrate. Platelet-rich plasma (PRP) and platelet suspension in PBS containing 1 mmol/L Ca\(^2+\) were obtained as described previously.\(^\)\(^9\)\(^-\)\(^11\) Human MNCs were isolated by a density-gradient centrifugation method as described previously.\(^17\) MNCs were suspended in 2 mL of PBS and were \(\geq\)90% pure; viability was confirmed by trypan blue exclusion.
Treatment of Platelets
Isolated platelets were stimulated with either thrombin (0.5 U/mL) or a protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA) (100 nmol/L). To examine the effects of α-tocopherol on intraplatelet α-tocopherol concentrations, platelets were treated with α-tocopherol (50, 100, or 500 μg/mL) or its vehicle (polyethylene 60-hydrogenated castor oil) for 10 minutes before platelet stimulation. In other experiments, the effects of the PKC inhibitor staurosporine on platelet functions were examined.

Flow Cytometric Determination of P-Selectin and Glycoprotein GPIb (CD42b)
We examined P-selectin expression on platelets by using flow cytometry, as described. Fifty-microliter aliquots of platelet suspension (2×10⁶ cells/μL) were incubated under various conditions, as mentioned above. After incubation, platelets were fixed in 1% paraformaldehyde in PBS (pH 7.4) and washed twice with PBS containing 10% fetal bovine serum (FBS). Platelet suspensions were then treated with a phycoerythrin (PE)-conjugated anti-human P-selectin monoclonal antibody (mAb) (1:10 dilution) (BD Pharmingen), and were kept at 4°C for 30 minutes. For control staining, mouse IgG1, with PE (1:10 dilution) was added to the cell suspension. As a control platelet surface molecule that is constituatively expressed, glycoprotein GPIb (CD42b) was also analyzed by using an FITC-conjugated anti-CD42b mAb (BD Pharmingen) or isotype-matched mouse IgG1.

Platelet PKC Activity Assay
Platelets were suspended in modified Tyrode’s/HEPES buffer, pH 7.4, containing (in mmol/L) NaCl 134; KCl 2.9; NaHCO₃ 12; CaCl₂ 1; HEPES 5; glucose 5; and protease inhibitors (Boehringer Mannheim). Platelets were first treated with graded concentrations of α-tocopherol (10, 50, 100, and 500 μg/mL) or vehicle for 10 minutes, followed by treatment with PMA (100 nmol/L) for 5 minutes. Cells were then lysed by sonication for 2 seconds × 8 on melting ice, with the use of an ultrasonic cell disruptor (Ultrasonics). The cell lysates were centrifuged at 100 000g for 40 minutes at 4°C to separate the membrane fraction. The membrane fraction was resuspended in 200 μL of glycerol/Tris buffer with 0.1% NP-40 and was subjected to PKC activity assay.

The PKC activity was measured by a method described by Toomik et al. PKC activity was standardized with protein concentration of each sample. The relative PKC activity was expressed as fold increase from the control nonsupplemented sample. Protein concentrations were determined by a Protein Assay ESL kit (Boehringer Mannheim), with BSA used as a standard.

Platelet-MNC Interaction
We examined whether α-tocopherol inhibited P-selectin-mediated platelet-MNC interaction. Platelets were incubated under various conditions, as mentioned above. Treated platelets were mixed with nonsupplemented MNCs. Cell mixtures were then examined under light microscopy, and the degree of platelet-MNC rosetting formation was analyzed.

We also used a 2-color flow cytometry analysis to assess platelet-MNC interaction. The mixtures of platelets and MNCs were fixed with 1% paraformaldehyde in PBS. After gentle washing with PBS containing 10% FBS, cell mixtures were incubated with both an FITC-conjugated anti-human CD45 mAb (leukocyte marker) and a PE-conjugated, nonblocking anti-human P-selectin mAb (platelet marker) for 30 minutes at 4°C. After incubation, samples were washed twice with PBS containing 10% FBS, fixed again in 1% paraformaldehyde, and analyzed by flow cytometry. Particles positively stained with both FITC and PE (CD45⁺/P-selectin⁺) were defined as platelet-MNC-interacted particles.

Platelet Aggregation
We also examined the effects of α-tocopherol on platelet aggregation. Washed human platelet suspensions (2×10⁶ cells/μL) were prepared in PBS containing 1 mmol/L Ca²⁺. Platelets were then incubated under various conditions, as mentioned above, with continuous stirring during the experiments at 37°C. Platelet aggregation was analyzed with the use of an 8-channel platelet aggregometer (MC Medical).

Intraplatelet α-Tocopherol Concentration After In Vitro Treatment
We analyzed intraplatelet α-tocopherol concentrations in gel-filtered platelets after in vitro treatment with α-tocopherol (50 to 100 μg/mL). After the treatment of PRP with α-tocopherol (50 and 100 μg/mL), gel-filtered platelets were immediately prepared, and platelet α-tocopherol concentrations were measured by HPLC.

Ex Vivo Experiments in Healthy Human Subjects
Oral Supplementation of α-Tocopherol
We examined whether ex vivo platelet P-selectin expression, platelet-MNC interaction, and platelet aggregation would be modified after oral supplementation of α-tocopherol in healthy human subjects. Ten healthy adult volunteers who did not smoke and were not taking any medications received oral α-tocopherol (300 mg/d, Eizai) supplementation for 3 weeks. This protocol was approved by the institutional review board, and informed consent was obtained from each subject.

Plasma and Intraplatelet α-Tocopherol Concentrations
Plasma concentrations of α-tocopherol were analyzed before and after an oral supplementation of α-tocopherol by means of HPLC (expressed as μg/mL, plasma). To analyze intraplatelet concentrations of α-tocopherol, gel-filtered platelets were obtained by passing PRP through a 2% Sepharose (Sepharose 2B, Pharmacia Biotech) column equilibrated with Ca²⁺-free Tyrode’s buffer (in mmol/L; NaCl 137; KCl 2.7; NaHCO₃ 12; MgCl₂ 1; glucose 5.5). α-Tocopherol concentrations in the gel-filtered platelets were measured by HPLC (expressed as ng/10⁵ platelets).

Flow Cytometric Determination of P-Selectin Expressed on Platelets
Before and after oral supplementation, flow cytometry was performed to analyze ex vivo platelet P-selectin expression induced by thrombin (0.5 U/mL) or PMA (100 nmol/L). Flow cytometric examination was performed as described above.

Platelet-MNC Interaction and Aggregation
Before and after oral α-tocopherol supplementation, we used 2-color immunofluorescence flow cytometry to assess platelet-MNC interaction ex vivo, as described above. We also examined ex vivo platelet aggregation in response to thrombin (0.001 to 0.5 U/mL) or PMA (10 to 100 nmol/L), as described above.

Statistical Analysis
Results are expressed as mean±SEM. All data were subjected to 1-way ANOVA, followed by the Fisher’s test for comparison of any two means. A value of P<0.05 was considered statistically significant.

Results
Effects of α-Tocopherol on P-Selectin Expression
In nonsupplemented platelets, P-selectin expression was minimal, whereas it was markedly enhanced after stimulation with either thrombin (0.5 U/mL) or PMA (100 nmol/L). Pretreatment with α-tocopherol (50, 100, and 500 μg/mL) significantly inhibited thrombin-induced or PMA-induced P-selectin expression (Figure 1). Control mouse IgG did not bind to platelets. Stauroporine (100 nmol/L), a potent PKC inhibitor, also inhibited either thrombin-stimulated or PMA-stimulated P-selectin expression. In contrast, CD42b (GP1b) was constitutively expressed on platelets regardless of the treatments with either thrombin (0.5 U/mL) or different
concentrations of α-tocopherol (50, 100, 500 μg/mL). Percent positive expression for CD42b was >90% in platelets with all conditions (FACS histograms not shown).

Effects of α-Tocopherol on Platelet PKC Activity
We examined the effects of α-tocopherol on PKC activity in human platelets. PMA (100 nmol/L) stimulated the membrane PKC activity by 2.5-fold (P<0.01), peaking at 5 minutes after stimulation. α-Tocopherol (10, 100, 500 μg/mL), added 10 minutes before PMA stimulation, significantly inhibited PMA-induced PKC activation in a concentration-dependent manner (Figure 2).

Effects of α-Tocopherol on Platelet-MNC Interaction
We next examined whether α-tocopherol functionally inhibited platelet-MNC adhesion. When platelets were stimulated with either thrombin (0.5 U/mL) or PMA (100 nmol/L), platelet-MNC rosette formation was markedly enhanced compared with nonstimulated platelets. α-Tocopherol (500 μg/mL) significantly suppressed thrombin-induced or PMA-induced platelet-MNC interaction. The PKC inhibitor staurosporine (100 nmol/L) also attenuated the platelet-MNC interaction induced by thrombin or PMA, mimicking the effects of α-tocopherol (Figure 3, A and B).

We quantified the effects of α-tocopherol on thrombin-induced platelet-MNC interaction by flow cytometry (Figure 3C). Thrombin (0.5 U/mL) enhanced the formation of the particles double-positive for FITC and PE (ie, platelet-MNC adherence particles) compared with nonstimulated platelets. Treatment of platelets with α-tocopherol (50, 100, 500 μg/mL) significantly inhibited platelet-MNC interaction in a concentration-dependent manner (59%, 39%, and 24%, respectively) (Figure 3C). Thus, both the microscopic evaluation (rosette formation) and flow cytometric analysis revealed that α-tocopherol inhibited platelet-MNC interaction.

Effects of α-Tocopherol on Platelet Aggregation
We examined the effects of α-tocopherol on platelet aggregation. α-Tocopherol (10, 100, and 500 μg/mL) inhibited platelet aggregation induced by either thrombin or PMA (Figure 4, A and B). Staurosporine (100 nmol/L), a PKC inhibitor, also inhibited platelet aggregation by thrombin (0.5 U/mL) or PMA (100 nmol/L) (Figure 4, C and D). As summarized (Figure 4, E and F), α-tocopherol (50, 100, and 500 μg/mL) or staurosporine (100 nmol/L) attenuated thrombin-induced (0.5 U/mL) or PMA-induced (100 nmol/L) platelet aggregation.

Intraplatelet α-Tocopherol Concentrations
Because α-tocopherol is lipophilic, relatively high concentrations of extracellular α-tocopherol (ie, 50 and 100 μg/mL) was needed to achieve sufficient elevation of intraplatelet α-tocopherol concentrations after short-term incubation in vitro. We analyzed intraplatelet α-tocopherol concentrations in gel-filtered platelets after in vitro treatment of platelets with α-tocopherol. Intraplatelet contents of α-tocopherol were 32±3 and 58±7 ng/10⁷ platelets after 10 minutes of in vitro treatment of platelets with 50 and 100 μg/mL of α-tocopherol, respectively.

Ex Vivo Experiments in Healthy Human Subjects
Plasma and Intraplatelet α-Tocopherol Concentrations
Finally, we examined the effects of clinically relevant doses of α-tocopherol on P-selectin expression in human subjects. Oral α-tocopherol supplementation (300 mg/d for 3 weeks) resulted in increased plasma concentrations of α-tocopherol from 8.3±0.2 to 16.8±1.2 μg/mL (P<0.01) (n=10). Intraplatelet α-tocopherol levels were also significantly increased from 6.1±0.5 to 29±3.9 ng/10⁷ platelets (P<0.01) (n=10). Either plasma or intraplatelet concentrations of β- and γ-tocopherols, inactive forms of tocopherol, were unchanged. Overall, intraplatelet concentrations of α-tocopherol were similar between in vitro incubation with high doses of α-tocopherol (50 μg/mL) and ex vivo...
Figure 3. A, Platelet-MNC rosette formation. a, Almost no rosette formed by control nonstimulated platelets and MNCs. b, Thrombin (0.5 U/mL) increased the formation of rosettes. c, α-Tocopherol (500 μg/mL), and d, staurosporine (100 nmol/L) attenuated thrombin-induced rosette formation. e, PMA (100 nmol/L) increased the formation of rosettes. f, α-Tocopherol (500 μg/mL), and g, and staurosporine (100 nmol/L) attenuated PMA-induced rosette formation. Original magnification ×400. B, Percentages of rosette-positive MNCs to total MNCs. Thrombin and PMA significantly increased the formation of rosettes. Both α-tocopherol and staurosporine inhibited rosette formation stimulated by thrombin or PMA. C, Dots located in the upper right corner of graphs (FITC- and PE-positive) represent platelet-MNC interaction. b, Thrombin (0.5 U/mL) platelet-MNC interaction compared with nonstimulated platelets (a). c, d, and e, α-Tocopherol (50, 100 and 500 μg/mL) inhibited the platelet-MNC interaction in a concentration-dependent manner.
conditions after oral α-tocopherol supplementation (300 mg/d for 3 weeks).

**Effects of Oral Supplementation of α-Tocopherol on Agonist-Induced Platelet P-Selectin Expression**

Before supplementation of α-tocopherol, both thrombin (0.1 and 0.5 U/mL) and PMA (10 and 100 nmol/L) markedly stimulated P-selectin expression on platelets (Figure 5A). The thrombin- or PMA-induced P-selectin expression was significantly attenuated after oral supplementation of α-tocopherol for 3 weeks (Figure 5A).

**Effects of Oral Supplementation of α-Tocopherol on Platelet-MNC Interaction Ex Vivo**

Before supplementation of α-tocopherol, both thrombin (0.001 to 0.5 U/mL) and PMA (1 to 100 nmol/L) significantly stimulated platelet-MNC interaction in a concentration-dependent manner (Figure 5B). The thrombin- or PMA-induced platelet-
Before supplementation of α-tocopherol, both thrombin (0.001 to 0.5 U/mL) and PMA (1 to 100 nmol/L) significantly stimulated platelet aggregation (Figure 5C). The thrombin- or PMA-induced platelet aggregations were significantly attenuated after oral supplementation of α-tocopherol for 3 weeks (Figure 5C).

**Discussion**

**Major Findings of the Present Study**

α-Tocopherol inhibited agonist-induced P-selectin expression on human platelets and thereby inhibited platelet-MNC interaction. α-Tocopherol did not influence the expression of another platelet cell surface molecule CD42b. α-Tocopherol inhibited agonist-induced platelet aggregation. Inhibitory actions of α-tocopherol against platelet P-selectin expression, platelet-MNC interaction, and aggregation were mimicked by the PKC inhibitor staurosporine. α-Tocopherol prevented PMA-stimulated PKC activation in human platelets. Oral α-tocopherol supplementation (300 mg/d for 3 weeks) in healthy human subjects attenuated ex vivo platelet P-selectin expression, platelet-MNC interaction, and platelet aggregation.

Because P-selectin on the activated platelet surface is a key adhesion molecule for initiating thrombus formation in vivo, the present findings provide new insights into the antithrombotic mechanisms of α-tocopherol.

α-Tocopherol is an antioxidant, and this property has been considered to be a main property of the antiatherogenic efficacy of α-tocopherol. However, it is unlikely that α-tocopherol acts solely as an antioxidant because the rate constant of the interaction of superoxide with α-tocopherol is 5 orders of magnitude lower than that of superoxide with superoxide dismutase or nitric oxide. Recent studies have shown that α-tocopherol has the ability to inhibit the PKC activity. For example, Boscobonik and co-workers showed that α-tocopherol inhibits vascular smooth muscle cell proliferation by suppressing PKC activity.

Geng and coworkers previously reported that PKC activation is a key event for rapid P-selectin expression on platelets. Thrombin, a potent stimulant for P-selectin, also activates phosphatidyl inositol (PI) turnover and diacylglyceride formation and thus activates PKC. Moreover, Hannun et al. showed that PKC activation is a necessary and common event for platelet activation. Conversely, PKC inhibitors have been shown to prevent the rapid P-selectin expression on platelets during agonist-induced activation. In the present study, α-tocopherol suppressed platelet P-selectin expression stimulated by either PMA or thrombin (Figure 1). α-Tocopherol attenuated the increase in platelet PKC activity by PMA in a concentration-dependent manner. Taken together, α-tocopherol probably inhibited rapid P-selectin expression by suppressing the PKC-dependent signal transduction pathway. Our results are consistent with previous studies showing that α-tocopherol suppresses platelet P-selectin expression and plasma levels of soluble P-selectin and further clarifies potential mechanisms of those inhibitory actions. To confirm that the suppressive action of α-tocopherol on platelet surface molecules is P-selectin–
specific, we examined the effects of α-tocopherol on CD42b expression. The CD42b expression was not affected by the treatments with α-tocopherol, indicating that not all adhesion molecules on platelets were regulated by α-tocopherol treatment.

We next examined whether α-tocopherol would modulate functional aspects of P-selectin on the activated platelet surface. P-selectin on platelets mediates platelet binding to MNCs, which activates leukocytes.4,8 Weyrich and coworkers14 and Neumann and coworkers15 reported that when activated platelets adhere to leukocytes, they activated leukocytes to let them release inflammatory cytokines such as MCP-1 through a P-selectin–dependent manner. Nagata and coworkers16 also showed that platelets stimulated monocytes to release oxygen free radicals in a P-selectin–dependent manner. These studies support that P-selectin expressed on activated platelets plays prothrombotic and atherogenic roles at least in part by facilitating a platelet-leukocyte interaction. In the present study, α-tocopherol suppressed either PMA-stimulated or thrombin-stimulated platelet-MNC interactions. The antiadhesive effect of α-tocopherol was mimicked by staurosporine, a PKC inhibitor, indicating that the platelet PKC pathway may play a key role in the interaction between platelets and MNCs mediated by P-selectin.

Another important finding in the present study is that oral supplementation of an ordinary dose of α-tocopherol (300 mg/d for 3 weeks) in healthy humans prevented rapid P-selectin expression on platelets and related platelet-MNC interactions ex vivo. Because of the lipophilic nature of α-tocopherol, it may take at least several weeks for α-tocopherol to be incorporated into intact cells by oral administration.16-24 In the present study, α-tocopherol levels in both platelet-poor plasma and gel-filtered platelets were significantly increased by 2.5- and 5-fold, respectively, after oral α-tocopherol supplementation for 3 weeks (Figure 5, A and B). Our findings therefore suggest that oral supplementation of α-tocopherol in human subjects results in accumulation of sufficient amounts of α-tocopherol in platelet membranes to modulate platelet function, such as P-selectin expression, and thereby makes platelets resistant to agonist-induced stimulation.

In summary, α-tocopherol attenuated P-selectin expression on activated human platelets and thus inhibited the P-selectin–dependent function, platelet-MNC interaction. The mechanism probably was related to the inhibition of PKC activity in platelets. Since P-selectin is an important atherothrombogenic adhesion molecule, the present finding will provide us new insights into the mechanism by which dietary α-tocopherol inhibits thrombosis and atherogenesis and thereby reduces the risk of coronary artery diseases.

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