Background and Objectives. The combination of vitamin E with aspirin is becoming an attractive therapeutic approach to prevent thrombotic vascular accidents. In this study we investigated the capacity of vitamin E (50 and 100 µM) to enhance the antiplatelet effect of aspirin.

Design and Methods. The dose-response curves of platelet aggregation, dense body secretion, phospholipase C activation and calcium mobilization were measured in aspirin-treated platelets with and without added vitamin E (50 and 100 µM). The role of vitamin E in reducing platelet adhesion to collagen was also studied.

Results. We demonstrated that, in platelets incubated with 100 µM vitamin E, collagen-concentration (µg/mL) able to induce 50% of the maximal platelet aggregation and of the calcium mobilization was higher than in controls (11.6 versus 3.8 and 21.3 versus 9.8, respectively). Furthermore, 50 µM vitamin E reduced platelet adhesion to collagen by about 80%.

Interpretation and Conclusions. These data demonstrate that vitamin E can potentiate the antiplatelet activity of aspirin by inhibiting the early events of platelet activation pathways induced by collagen. This finding provides a rationale for combining aspirin and vitamin E to prevent thrombotic complications in atherosclerotic patients.

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Key words: platelets, vitamin E, aspirin, phospholipase C, collagen.

Vitamin E is an antioxidant agent which has been tested in large clinical trials to assess its potential usefulness in preventing cardiovascular complications secondary to atherosclerotic processes. The rationale for such a therapy is based on the assumption that oxidant stress plays a pivotal role in the progression of atherosclerotic lesions. Experimental studies showed that vitamin E reduces oxidation of low density lipoproteins (LDL), which seem to be crucial in the recruitment of macrophages and production of foam cells, but no relationship was observed between inhibition of LDL oxidation and prevention of experimental atherosclerosis.1

Platelets have a key role in the initiation and progression of atherosclerotic lesions, and antiplatelet drugs reduce the rate of cardiovascular death, myocardial infarction and stroke in patients with atherosclerotic disease.2 The interaction between platelets and collagen represents an essential event for the production of a hemostatic plug under physiologic and pathologic conditions. Circulating platelets rapidly adhere to newly exposed collagen fibers after endothelial damage and become activated to recruit additional platelets to the site of injury. The collagen-induced platelet activation results in shape change, granule secretion and conformational changes of integrin αIIbβ3, leading to a fibrinogen-dependent platelet-platelet interaction. All these mechanisms are still present in aspirin-treated platelets even though a higher collagen concentration is needed to induce platelet adhesion and activation.3

It has been previously demonstrated that vitamin E interferes with collagen-induced platelet activation in virtue of its ability to quench H2O2 production in human platelets;4 furthermore, the inhibitory effect of a scavenger of H2O2, such as catalase, was particularly evident in platelet aggregation when platelets were pretreated with aspirin and...
Vitamin E and aspirin in collagen stimulated platelets

stimulated with high collagen concentrations. The combination of vitamin E with aspirin has been confirmed to be more effective than aspirin alone in reducing the incidence of ischemic events although the underlying mechanism has not yet been defined.

In this study we incubated aspirin-pretreated platelets with vitamin E at concentrations usually found in peripheral circulation after oral supplementation in order to investigate whether vitamin E is able to enhance the inhibitory effect of aspirin in collagen-stimulated platelets. Furthermore we investigate whether vitamin E reduces the platelet-collagen interaction in aspirin-pretreated platelets.

Design and Methods

Materials

$^{32}$Pi, $^3$H oleic acid and $^3$H inositol 1,3,4 P$_3$ were obtained from Amersham (Arlington Heights, IL, USA). The Fura 2-AM came from Molecular Probes (Eugene, OR, USA), and the Sepharose 2B from Pharmacia (Uppsala, Sweden). Collagen, type 1, (Clifton, USA), and the Sepharose 2B from Whatman, (Clifton, USA), and the ODS 5 µM column (4.6×250 mm) was from Restek Corporation (Bellefonte, PA, USA). Bovine serum albumin, Hepes, acetyl salicylic acid (ASA), α-tocopherol (vitamin E), fibrinogen, inorganic pyrophosphatase, digito- tin, formaldehyde, ammonium formate, maleic acid, creatine phosphate (CP), creatine phosphokinase (CPK), and Arg-Gly-Asp-Ser (RGDS) were all purchased from Sigma Chemicals Co., (St. Louis, MO, USA).

Platelet preparation

Human blood was obtained from free healthy volunteers who were not taking any medication and anticoagulated with acid/citrate/dextrose. Platelet-rich plasma, obtained by centrifugation (15 min at 180 g), was recentrifuged (20 min at 800 g) to concentrate the platelets, and the pellet was resuspended in 0.5 vol. of autologous platelet-poor plasma.

Before platelet separation from plasma proteins, the platelet suspensions, utilized to detect calcium mobilization, inositol 1,3,4 P$_3$ formation and platelet adhesion to collagen, were incubated for 1 h at 37°C with 3 µM Fura 2-AM or with $^{32}$Pi 2 mCi/mL or with 1 mCi/mL $^3$H-oleic acid.

Platelets were separated from plasma proteins and from the excess of Fura 2-AM, $^{32}$Pi and $^3$H-oleic acid by gel filtration on Sepharose 2B using Ca$^{2+}$-free Tyrode's buffer containing 0.2% bovine serum albumin, 5 mM glucose and 10 mM Hepes, pH 7.35. After gel filtration the cell suspension was adjusted to a final concentration of 2×10$^8$ cells/mL.

In all the experiments vitamin E (50 and 100 µM) (dissolved in ethanol at a concentration of 10 mM) was added to platelets preincubated with 100 µM aspirin for 15 min at 37°C before addition of the agonist. The same amount of vitamin E solvent was added to the control samples. In order to obtain appreciable platelet responses, we first constructed a dose-response curve using different concentrations of collagen and measuring platelet aggregation (from 2 to 20 µg/mL), platelet secretion (2 to 20 µg/mL), inositol 1,3,4 P$_3$ formation (from 10 to 50 µg/mL) and Ca$^{2+}$ mobilization (from 4 to 40 µg/mL).

Extraction and measurement of α-tocopherol

One milliliter of a suspension of washed platelets, obtained from 10 healthy volunteers who were not taking any medication, was treated with α-tocopherol (50-100 µM) or left untreated. After 15 min incubation, the suspension was washed twice by centrifugation. The platelet pellet was then resuspended in 1 mL Tyrode’s buffer and precipitated with 1 mL iced methanol, vigorously shaken and then extracted by adding 2 mL n-hexane. The mixture was allowed to settle. The organic top layer was removed and dried under nitrogen; 200 µL of methanol were added to the dried extract, and the suspension was filtered through a 0.2 mm filter (Millipore Corp, Bedford, MA, USA).

A 50 µL aliquot was analyzed on an ODS 5 µM column and eluted with methanol/water (95:5, vol/vol) at a flow rate of 2 mL/min. Optical absorbance was measured with a Diode-Array Detector (Perkin-Elmer, Wellesley, MA, USA) at 240 and 290 nm; the retention time for α-tocopherol was calculated using tocopheryl-acetate as internal standard.

Platelet adhesion to collagen

To study platelet adhesion to collagen (50 µg/mL) without the interference of aggregation and activation induced by all the known agonists released from platelets after collagen stimulation, $^3$H-oleic acid-labeled platelets were preincubated with the cyclo-oxygenase inhibitor ASA (100 µM), with the ADP-removing system CP/CPK (20 mM and 50 U/mL, respectively), and with the fibrinogen/fibronectin antagonist RGDS (10 µM). Following this, the $^3$H-oleic acid-labeled platelet suspensions were incubated with collagen (50 µg/mL) for 1 min at...
37°C. The platelets that adhered to collagen were separated from those that did not by filtration through a 10 µm nylon mesh (Small Parts Inc., Miami, Florida, USA), and the disks were transferred to counting vials in order to measure the extent of platelet adhesion. We considered this platelet-collagen interaction specific for platelet adhesion to collagen as the procedure was performed in the absence of aggregation by antagonizing all the known receptors for extracellular agonists without affecting events that could alter intracellular metabolism.

Platelet aggregation

The platelet aggregation induced by collagen (2 to 20 µg/mL) was evaluated according to Born's method in four sample PACKS 4 (Helena Laboratories, Beaumont, Texas, USA) at 37°C using sili-
cized glass cuvets under continuous stirring at 1,000 rpm. Platelet aggregation was expressed as the mean of the percentage of the maximum obtained in each experiment. Fibrinogen (1 mg/mL) was added before the agonist.

Platelet secretion

The platelet secretion induced by collagen (2 to 20 µg/mL) was evaluated by measuring the release of ATP. The activation of platelets was stopped after 2 min with formaldehyde/EDTA according to a previously described procedure. After centrifugation at 10,000 × g for 30 sec, the ATP concentration in the supernatant was measured using a LKB 1251 luminometer (Pharmacia) after the addition of luciferin (40 mg/mL) and luciferase (880 U/mL). The results were expressed as the percentage of ATP release relative to the total ATP present in lysed cells.

Phospholipase C activation

Phospholipase C activation induced by collagen (10 to 50 µg/mL) was measured as inositol 1,3,4 P₃ (IP₃) formation obtained by HPLC separation from labeled neutralized platelet extracts. The inositol 1,3,4 P₃ production was evaluated 1 min after the platelet stimulation by collagen. The collagen activation of the [³²P]-labeled platelets (1x10⁹ cells/mL) resuspended in phosphate free Tyrode's buffer was stopped by means of perchloric acid (0.44 N). The neutralized platelet extracts were treated overnight with Zn²⁺ pyrophosphatase 20 U/mL in the presence of Tris-maleic buffer 0.1 M, pH 6.5 and then passed on a HPLC column which was eluted with a 50 min linear gradient of water as the first buffer, and ammonium formate 1.5 M, pH 3.75 as the final buffer. Inositol peaks were detected using a dual channel (¹H-⁳²P) HPLC radioactivity detector FLO-ONE A100 (Radiomatic, Camberra Company, Tampa, USA) using [³H]-inositol 1,3,4 P₃ as the pure standard. The results were expressed as a percentage of the maximum ∆ cpm of ³²P-IP₃ recovered after platelet activation.

Ca²⁺ mobilization

Ca²⁺ mobilization was measured with the fluorimetric probe FURA-2. FURA-2 fluorescence signals were detected with a SFM 25 fluorimeter (Kontron, Zurich, Switzerland) set at 340 nm excitation and 510 nm emission under continuous stirring and at 37°C. In order to convert fluorescence measurements into Ca²⁺ concentrations, Fₘᵢₙ was determined after the addition of digitonin (50 µM) in the presence of EGTA (2 mM) and Tris Base (20 mM); Fₘₐₓ was measured by the addition of excess CaCl₂ (10 mM). Cytosolic free calcium concentrations were calculated according to a previously described method, using a Kd of 224 nM. The results were expressed as the ∆ of cytosolic calcium concentration between stimulated and unstimulated platelets obtained in each experiment. At a concentration of 100 µM, vitamin E does not reduce the basal fluorescence in FURA-2-loaded platelets.

Statistical analysis

Data are reported as means ± SEM. Comparisons between variables were analyzed by Student’s t test for unpaired data. Results were considered statistically significant at the p<0.05 level.

Results

Vitamin E platelet concentrations were significantly increased after the cells had been incubated for 15 min at 37°C with vitamin E 50 µM and 100 µM; α-tocopherol concentrations obtained from the platelet extracts were 1.4±0.6 nmoles/10⁹ cells and 2.1±0.9 nmoles/10⁹ cells, respectively (basal values 0.23±0.15 nmoles/10⁹ cells). These values are similar to the α-tocopherol concentrations found in platelets obtained from healthy volunteers taking 400-800 IU/day of vitamin E supplementation.

Vitamin E, at a concentration of 50 µM or 100 µM, inhibited platelet adhesion to collagen. Aspirinated platelets were pretreated with the ADP scavenger system, CP/CPK, to avoid the interference of ADP release, and with the antagonist of the fibrinogen binding site, RGDS, and activated for 1 minute with 50 µg/mL of collagen. When platelets were preincubated with 50 µM vitamin E, platelet adhesion to collagen was reduced by about 80%, while incubation with 100 µM almost completely
suppressed platelet adhesion (Figure 1).

Furthermore, vitamin E inhibited the aggregation induced by collagen (2-20 µg/mL) of aspirin-treated platelets: a dose-response curve (Figure 2) showed that, in the presence of vitamin E (50 and 100 µM), a higher collagen concentration was needed to obtain 50% of maximal platelet activation (7.77±0.38 µg/mL and 10.82±0.46 µg/mL, respectively versus 4.25±1.03 µg/mL of untreated aspirinated-platelets; p<0.0001).

Platelet secretion induced by collagen (2-20 µg/mL) was inhibited by vitamin E both at 50 µM and, to a greater extent, at 100 µM. Figure 3 shows the dose-response curves of ATP release from collagen-stimulated aspirinated-platelets, treated and untreated with vitamin E. Vitamin E (50 µM and 100 µM) reduced the collagen concentration able to induce 50% of maximum ATP release in aspirin-treated platelets (7.8±2.1 µg/mL, p<0.005, and 11.6 ±3.8 µg/mL, p<0.005, respectively versus 3.8±1.1 µg/mL obtained in untreated platelets).

Phospholipase C activation was measured as inositol 1,3,4 P₃ production. Figure 4 illustrates the means of four dose-response curves of the percentage of inositol 1,3,4 P₃ produced compared to the maximum obtained in each experiment, in vitamin E (50 µM and 100 µM) treated and untreated platelets. Fifty percent of maximal IP₃ formation was obtained with collagen 12.5±1.47 µg/mL, whereas collagen concentrations of 15.3±0.81 µg/mL (p<0.05) and 20.5±1.14 µg/mL (p<0.05) were necessary in aspirinated-platelets treated with 50 µM and 100 µM vitamin E, respectively.

Collagen-induced Ca²⁺ mobilization was also inhibited by vitamin E (50-100 µM), in aspirin-treated platelets. In the presence of high doses of vitamin E, a greater collagen concentration was needed to obtain 50% of maximal calcium mobility.

Figure 1. Effect of Vitamin E (50 and 100 µM) on the collagen (50 µg/mL) adhesion of [³H]oleic-acid-labeled platelets. Platelets were incubated with vitamin E for 15 min at 37°C. The cells were pretreated with ASA (100 µM), RGDS (120 mM) and the ADP scavenger system CP/CPK (20 µM and 50 U/mL respectively) before collagen stimulation. Mean ± SEM from 5 experiments (*p<0.001 using Student’s t test for unpaired data).

Figure 2. Dose-response curves of collagen (2-20 µg/mL) induced aggregation of platelets added with and without (open dots), 50 µM (black dots) and 100 µM (black squares) vitamin E (15 min of incubation at 37°C). The points used to build the dose-response curves represent the mean ± SEM of the % of maximum obtained from 7 different experiments. The mean of the maximum was 88±9%.

Figure 3. Dose-response curves of ATP release from collagen-stimulated aspirinated platelets in the absence (open dots) and in presence of vitamin E 50 µM (black dots) and 100 µM (black squares). The points used to build the dose-response curves represent the means±SEM of the % maximum obtained from 7 different experiments. The mean of the maximum was 64±12.8.

Figure 4. Dose-response curves of collagen (2-20 µg/mL).
lization as shown in dose-response experiments (Figure 5). In fact, $9.8 \pm 0.35 \, \mu g/mL$ collagen was necessary to obtain 50% of maximal calcium mobilization in aspirin-treated platelets, whereas in platelets treated with 50 $\mu M$ and 100 $\mu M$ vitamin E the collagen concentration needed to obtain 50% of maximal calcium mobilization rose to $15.6 \pm 0.89 \, \mu g/mL$ and $21.3 \pm 0.68 \, \mu g/mL$, respectively, showing a statistical significance of $p < 0.0001$ in both conditions.

Discussion

The oxidative hypothesis of atherosclerosis is based on the fact that LDL accumulates within the vessel wall upon oxidative changes; this allows LDL to be indefinitely taken up by macrophages. Antioxidant treatment can reduce LDL uptake by macrophages, thus interrupting the progression of atherosclerosis and its complications. Antioxidant molecules may also be useful because they can interfere with platelet function by inhibiting collagen-induced platelet activation.14 Platelet activation is a fundamental event for thrombus formation. In fact, rupture of the plaque and the subsequent exposure of the subendothelium allow platelet adhesion to collagen, platelet activation and finally thrombus formation. Collagen has been identified as the most thrombogenic macromolecule present in the extracellular matrix underlying the subendothelium15 as it provides an important site for platelet adhesion.16 Beside supporting adhesion, collagen also induces platelet activation, thromboxane A2 production and granule secretion leading to platelet aggregation and subsequently to thrombus formation.

An in vitro study demonstrated that catalase inhibits all the biochemical pathways leading to collagen-induced platelet aggregation by quenching platelet production of $H_2O_2$, an oxidant species that contributes to platelet activation induced by collagen.5

Studies in humans investigated the capacity of vitamin E to interfere with platelet function. Vitamin E, an antioxidant molecule, has the same effect as catalase in that it reduces $H_2O_2$ production by activated platelets. Accordingly, we demonstrated that vitamin E administered at a daily dosage of 600 mg significantly inhibited collagen-induced platelet aggregation and platelet $H_2O_2$ production.4

The present study reports that concentrations of vitamin E as low as 50 and 100 $\mu M$ are able to inhibit aggregation and secretion of aspirin-treated platelets stimulated with high concentrations of collagen.
In addition, vitamin E was able to inhibit platelet adhesion to collagen, a fundamental event occurring during thrombus formation. To the best of our knowledge this is the first evidence that vitamin E reduces platelet adhesion to collagen without any interference by ADP release and thromboxane A2 production. This finding is consistent with previous studies showing a potent antiadhesive effect after 400-600 IU/day of oral vitamin E supplementation, but in these works both the amplification pathways were not inhibited. As our results were obtained in aspirinated platelets it is possible to speculate that vitamin E strongly inhibits a cyclooxygenase-independent early event of platelet activation such as adhesion to collagen. This hypothesis is also supported by the evidence that the main mechanisms leading to granule secretion and aggregation, such as inositol P3 formation and calcium mobilization, which are independent of thromboxane A2 production, were inhibited by 50 and 100 µM vitamin E in the aspirinated platelets.

Freedman et al. reported that vitamin E inhibits ADP-induced platelet activation throughout the inhibition of phosphokinase C activity; however, our results suggest that vitamin E inhibits collagen-induced platelet activation independently of inhibition of the phosphokinase C pathway, as platelet adhesion, phospholipase C activation and calcium mobilization induced by collagen were not inhibited by phosphokinase C inhibitors. This finding provides an insight into the mechanism of cyclooxygenase-independent inhibition of platelet aggregation elicited by vitamin E and also provides evidence on the role of H2O2 as a second messenger in collagen-induced platelet aggregation. As the concentration of vitamin E able to inhibit platelet H2O2 release can be achieved in plasma by oral supplementation of 600 IU/die, our data suggest that the combination of aspirin and vitamin E may be useful for the treatment of progression of atherosclerosis in patients with cardiovascular disease by inhibiting platelet interactions with the vessel wall and consequent platelet activation leading to thrombus evolution. In conclusion, we demonstrate that vitamin E enhances the antiplatelet activity of aspirin by inhibiting an early event of collagen-induced platelet activation. This finding provides a rationale for combining aspirin and vitamin E in experimental studies on the prevention of thrombotic complications in atherosclerotic patients.

Contribution and Acknowledgments
AC and FV designed the study, performed the analyses and wrote the paper. AC, FMP, and PPG performed and critically reviewed the experimental procedure concerning the platelet-collagen interaction. PP, LL and GF performed and critically reviewed the experimental procedures related to the extraction and measurement of vitamin E. The order of the authors reflects their contribution to the paper.

Disclosure
Conflict of interest: none.
Redundant publications: no substantial overlapping with previous paper.

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**PEER REVIEW OUTCOMES**

**Manuscript processing**

This manuscript was peer-reviewed by two external referees and by Professor Carlo Balduini, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor Balduini and the Editors. Manuscript received December 4, 2001; accepted February 15, 2002.

**What is already known on this topic**

Both aspirin and vitamin E are known to inhibit in vitro platelet activation induced by collagen. However, the effect of the association has never been investigated.

**What this study adds**

Present study demonstrates that vitamin E potentiates the inhibitory effect of aspirin by reducing inositol triphosphate formation.

**Potential implications for clinical practice**

Clinical studies are required to evaluate whether the association of vitamin E and aspirin is better than aspirin alone for the prevention of thrombotic events deriving from exposure of subendothelial collagen.