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# Inhibition of cyclooxygenase-independent platelet aggregation by low vitamin E concentration

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**Summary**

Platelet aggregation induced by threshold concentrations of agonists such as collagen, PAF or epinephrine was inhibited in vitro by 100 $\mu$M aspirin but was restored by stimulating platelets with high concentrations of collagen, PAF or by a combination of epinephrine and PAF. Incubating aspirin-treated platelets with 50–100 $\mu$M vitamin E or vitamin E acetate inhibited platelet aggregation by high concentrations of collagen and PAF and by the combination of epinephrine and PAF; platelet thromboxane $A_2$ formation was less than 10% in samples incubated with 100 $\mu$M aspirin. Apyrase, added to aspirin-treated platelet, did not influence platelet aggregation induced by epinephrine and PAF. The present study suggests that concentrations of vitamin E as low as 50–100 $\mu$M inhibit cyclooxygenase-independent platelet aggregation when combined with an inhibitor of the arachidonate pathway.

Key words: Aspirin; Vitamin E; Platelet aggregation; Thromboxane $A_2$

## Introduction

Vitamin E is an antioxidant agent which prevents lipid peroxidation in vitro and in vivo [1–3]. It probably modulates platelet function since animals with a vitamin E-deficient diet show platelet hyperaggregation probably dependent on increased cyclooxygenase pathway activation [4]. Vitamin E has also been tested in human plasma where a concentration of about 1 mM inhibits platelet aggregation [5]. These findings are of scientific interest since they suggest the possible role of vitamin E in the modulation of platelet function; however, they are of no clinical value because a concentration of 1 mM vitamin E cannot be achieved in human blood by vitamin E supplementation [6]. This study reports on experimental models where low concentrations of vitamin E, relatively close to those found in human blood [6], inhibit platelet aggregation.

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## Materials and methods

The influence of vitamin E on platelet aggregation was tested in vitro in samples incubated with and without 100 μM aspirin, a cyclooxygenase inhibitor [7]. Blood samples mixed with 0.129 M sodium citrate (ratio 9 : 1) were taken from healthy subjects (aged 25–40 years) who had not ingested any drug interfering with platelet function in the previous 15 days, and gave informed consent to participate in the study. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described [8]; PRP was reconstituted at constant platelet count ($250 \times 10^9$ per liter). Platelet aggregation [9] was induced by threshold concentrations (TC) of PAF (3–37.5 nM), collagen (1–3 μg/ml) or epinephrine (0.75–2 μM), and expressed as the light transmission (LT) difference between PRP and PPP 3 min after adding the aggregating agents. The difference in LT between PRP and PPP was taken as 100% and the change in LT when platelets were aggregated expressed as a percentage. TC of agonist was defined as the lowest doses which induced at least 50% increase in light transmission. PRP was incubated 5 min at 37 °C with 100 μM aspirin or 100 μM vitamin E or 100 μM aspirin plus 50–100 μM vitamin E and stimulated with 10-fold TC of PAF or 10-fold TC of collagen. Platelet aggregation by PAF or collagen was also studied in samples without aspirin or vitamin E, as controls. In the latter, ethanol was added to PRP in a ratio of 1 : 500, which does not influence platelet aggregation. In another set of experiments, the cyclooxygenase independent platelet aggregation was investigated using a pair of aggregating agents. Platelet aggregation was first studied in PRP incubated with and without 100 μM aspirin and then stimulated with TC of PAF or TC of epinephrine. Platelet aggregation by TC of PAF plus TC of epinephrine (the latter being added 15 s before PAF) was studied in aspirin-treated samples with and without 100 μM vitamin E or 100 μM vitamin E acetate. In order to verify if in our model the cyclooxygenase-independent platelet aggregation was due to ADP pathway activation, we studied platelet aggregation in aspirin-treated PRP incubated with apyrase, a known scavenger of ADP [10]. Aspirin-treated (100 μM) PRP was incubated at 37 °C with and without 50 μg/ml apyrase and stimulated with TC epinephrine plus TC PAF.

*$TxA_2$ production*

Three min after the addition of the aggregating agents the reaction was stopped with absolute methanol, the sample was centrifuged for 2 min at $4000 \times g$ and the supernatant stored at −20 °C. $TxA_2$ was measured by its stable metabolite $TxB_2$ ($TxB_2$ RIA-Kit, New England Nuclear).

*PRP vitamin E concentration*

Saponification, extraction and HPLC analyses were done according to the Hatam method [11], partially modified by us. 150 μl PRP was transferred to a 10-ml screw-cap tube and mixed with 850 μl 70 mM ascorbic acid/1.25 mM EDTA; 1.0 ml absolute ethanol was then added and again the solution was gently mixed. 150 μl saturated KOH was added and the tubes were incubated for 30 min in a 50 °C water bath. After cooling on ice, 4.0 ml hexane containing 1.13 M BHT was added, the tubes were shaken vigorously for 3 min and the phases separated by centrifugation. Aliquots (20 μl) were injected into the HPLC column; results are expressed as the average of duplicate extractions. A Perkin Elmer model 410 with a SEC-4 solvent environmental control was used. Signals from the LC-95 spectrophotometer were integrated by an LC-100 integrator. The analytical column was a reverse-phase 5 μBondapak C18, 4.6 × 150 mm (Millipore); the mobile phase was methanol/acetonitrile/tetrahydrofuran (50 : 45 : 5, v/v), at a flow rate of 1 ml/min. The HPLC peaks were obtained by monitoring at 295 nm. Pure α-tocopherol from 0.5 to 2 ng/ml in absolute ethanol as injected to provide a standard curve. The minimal amount of α-tocopherol that could be detected was 1 ng; retention time of α-tocopherol was 5.05 min. A recovery test was carried out using a plasmatic pool containing 0.506 mg/dl (13.6 μM); the recovery was 99.8 ± 4.92% and the variation coefficient was 4.9%.

*Materials*

PAF (Sigma Chemical Company, U.S.A.) was dissolved in chloroform/methanol (2 : 1), dried under nitrogen and stored at −70 °C until use.

PAF working solution was prepared by diluting evaporated aliquots with Hank's balanced salt solution. Epinephrine (STAGO) was dissolved in Michaelis buffer, ratio 1 : 10. Collagen was purchased from Menarini. Aspirin as its soluble lysine salt (Flectadol, Maggioni, Milan) was dissolved in double-distilled water. Vitamin E (DL-$\alpha$-tocopherol, Merck) and vitamin E acetate (Aldrich Chem.) were dissolved in absolute ethanol. Apyrase (Sigma) was dissolved in Hank's balanced salt solution. KOH, EDTA, ascorbic acid, ethanol, methanol, acetonitrile and tetrahydrofuran were purchased from E. Merck (Darmstadt, West Germany); BHT was purchased from Sigma Chemical Company (St. Louis, U.S.A.).

*Statistical analysis*

Results are expressed as mean ± SE. Statistical evaluation was performed using Student's $t$ test on related samples. $P$ values $< 0.05$ are taken as being statistically significant.

## Results

Vitamin E did not affect platelet aggregation by PAF, which, on the contrary, was inhibited by 100 $\mu$M aspirin (Fig. 1). 10 TC of PAF induced irreversible platelet aggregation in aspirin-treated platelets; this aggregation, which was independent of the activity of the cyclooxygenase pathway, was significantly inhibited by the addition of vitamin E (Fig. 1). Similar findings were observed with collagen-induced platelet aggregation; indeed platelet aggregation induced by low collagen concentrations was inhibited by aspirin, but was fully

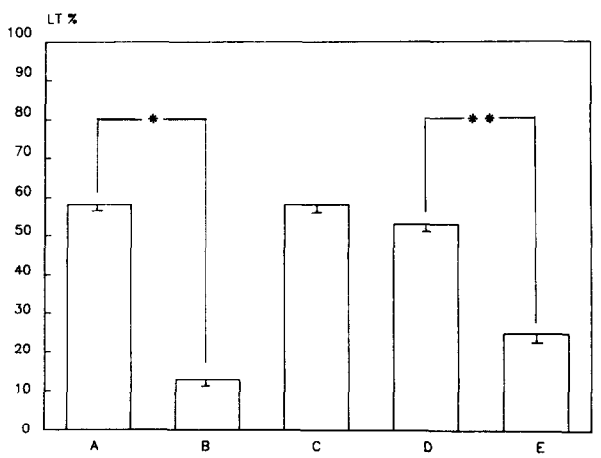


Fig. 1. Platelet aggregation (LT%) by low (threshold concentration) or high (10-fold threshold concentration) of PAF ($n = 4$). Threshold concentrations (TC) of PAF = 3–37.5 nM. ASA = aspirin, (A) TC PAF; (B) TC PAF + 100 $\mu$M ASA; (C) TC PAF + 100 $\mu$M vitamin E; (D) 10 TC PAF + 100 $\mu$M ASA; (E) 10 TC PAF + 100 $\mu$M ASA + 100 $\mu$M vitamin E. * $P < 0.001$, ** $P < 0.005$.

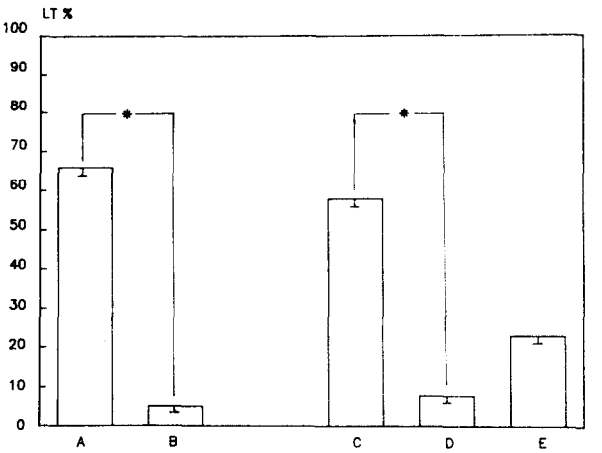


Fig. 2. Platelet aggregation (LT%) by low (threshold concentration) or high (10 fold threshold concentration) of collagen ($n = 4$). Threshold concentrations (TC) of collagen = 1–3 $\mu$g/ml. ASA = aspirin; (A) TC collagen; (B) TC collagen + 100 $\mu$M ASA; (C) 10 TC collagen + 100 $\mu$M ASA; (D) 10 TC collagen + 100 $\mu$M ASA + 100 $\mu$M vitamin E; (E, 10 TC collagen + 100 $\mu$M ASA + 50 $\mu$M vitamin E. * $P < 0.001$.

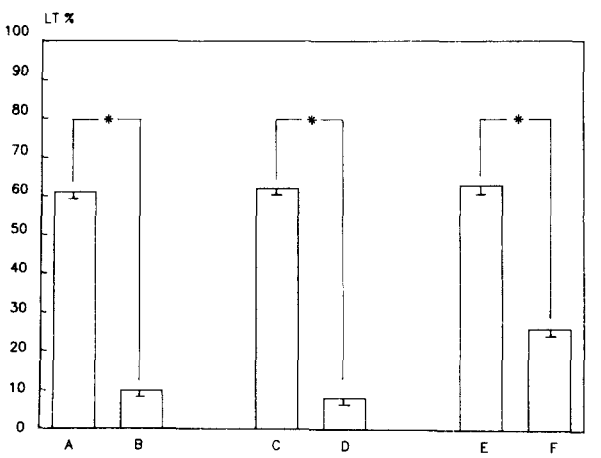


Fig. 3. Platelet aggregation (LT%) by threshold concentrations (TC) of PAF (3–37.5 nM) and/or TC of epinephrine (Ep; 0.75–2 $\mu$M) ($n = 4$). (A) TC PAF; (B) TC PAF + 100 $\mu$M ASA; (C) TC Ep; (D) TC Ep + 100 $\mu$M ASA; (E) TC PAF + TC Ep + 100 $\mu$M ASA; (F) TC PAF + TC Ep + 100 $\mu$M ASA + 100 $\mu$M vitamin E. * $P < 0.005$.

TABLE 1

PLATELET AGGREGATION (LT%) BY EPINEPHRINE PLUS PAF IN ASPIRIN-TREATED SAMPLES WITH AND WITHOUT 100 μM VITAMIN-E ACETATE OR 50 μg/ml APYRASE

| | LT% ($n = 4$) |
|---|---|
| PAF + Ep | 58 + /4 |
| PAF + Ep + 100 μM ASA | 56 + /6 |
| PAF + Ep + 100 μM ASA + 100 μM Vit-E ac. | 36 + /3 * |
| PAF + Ep + 100 μM ASA + 50 μg/ml apyrase | 56 + /5 |

* $P < 0.01$.

restored by increasing the concentration of collagen. The inhibition of platelet aggregation by vitamin E of aspirin-treated samples was dose-dependent (Fig. 2). Aspirin inhibited platelet aggregation by single agonists such as PAF or epinephrine but failed to prevent platelet aggregation in samples stimulated by epinephrine plus PAF; also in this case addition of vitamin E inhibited platelet aggregation (Fig. 3). Similar findings were obtained by using vitamin E acetate. In fact, at 100 μM concentration, vitamin E acetate significantly inhibited aspirin-treated PRP stimulated by epinephrine plus PAF (Table 1). No significant changes of platelet aggregation were observed in aspirin-treated samples incubated with apyrase and stimulated by a couple of agonists (Table 1).

Before the addition of exogenous α-tocopherol, PRP α-tocopherol concentration from 4 different donors was determined. Values of vitamin E ranged from 16.3 to 19 μM (17.4 ± 1.1 μM, mean ± SD).

Several controls were done to demonstrate the inhibition of the cyclooxygenase pathway in aspirin-treated samples stimulated with high concentrations or with a pair of aggregating agents; in all these different conditions we observed a marked inhibition of $TxA_2$ formation, which was always < 10% of the control (Fig. 4).

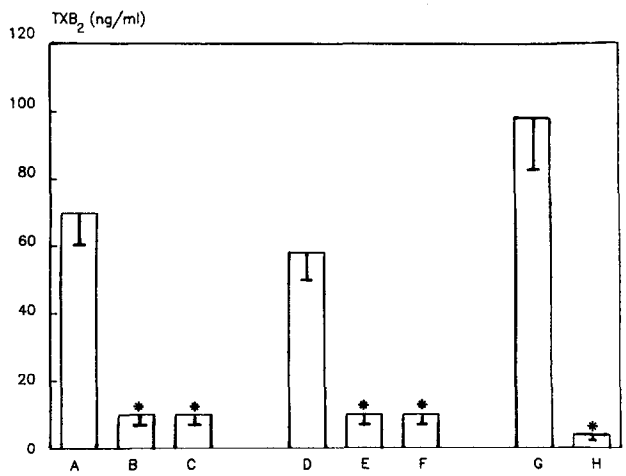


Fig. 4. Platelet $TxB_2$ (ng/ml) production by threshold concentrations (TC) or 10-fold TC of PAF or collagen and TC of epinephrine and PAF in PRP incubated with and without 100 μM aspirin (ASA) ($n = 8$). (A) = TC PAF; (B) = TC PAF + ASA; (C) 10 TC PAF + ASA; (D) TC collagen; (E) TC collagen + ASA; (F) 10 TC collagen + ASA; (G) TC PAF + TC epinephrine; (H) TC PAF + TC epinephrine + ASA. * $P < 0.001$.

## Discussion

In human blood α-tocopherol is carried by HDL and LDL [12]. Its concentration ranges from 12 to 59 μM, with mean values of approx. 20 μM [6,12], which are close to those we found in PRP. Vitamin E had been previously shown to inhibit the platelet release reaction and this effect has been attributed to its capacity of inhibiting cyclooxygenase or affecting platelet membrane fluidity [1]. However, these findings were obtained in vitro using high vitamin E concentrations such as 0.25–1 mM, which are much higher than those commonly found in human blood even after vitamin E supplementation [1,5,6]. Using lower concentrations of vitamin E or vitamin E acetate (in human blood much of vitamin E is in ester form), such as 0.05–0.1 mM, we inhibited platelet aggregation in aspirin-treated samples. The simultaneous inhibition of cyclooxygenase was crucial to demonstrate this vitamin E effect, suggesting that low vitamin E concentrations can inhibit cyclooxygenase-independent platelet aggregation. We used different stimuli which can activate platelet aggregation through a cyclooxygenase-independent mechanism. In the model we used containing low $Ca^{2+}$ concentration, collagen and PAF are recognized to activate platelets through arachidonic acid pathway and ADP release [13–15].

In accordance with other investigators [16], we observed a synergistic effect between epinephrine and PAF in inducing platelet aggregation. The enhancement by epinephrine of aggregation in response to other agonists is independent of thromboxane $A_2$ formation [17] and this is further supported by our experiments where aspirin-treated platelets fully aggregated in response to epinephrine and PAF. This effect had been detected in both high and low $Ca^{2+}$ media and is independent of the presence of fibrinogen in the medium [17]; some authors suggested that epinephrine-induced platelet aggregation needs traces of ADP [18] but the variability of data did not definitively support a role for ADP in epinephrine-induced platelet aggregation [17]. However, in our model ADP does not seem to play a major role since apyrase, a scavenger of ADP, did not influence cyclooxygenase-independent platelet aggregation induced by a couple of agonists. The mechanism(s) by which vitamin E inhibits cyclooxygenase-independent platelet aggregation needs to be further investigated. This effect should not be dependent on its antioxidant property since both vitamin E and its oxidized form, vitamin E quinone, inhibit platelet aggregation, the secretory release reaction and the cyclooxygenase pathway [1]. Interesting results have been obtained by Steiner and Mower [1] who demonstrated that vitamin E decreased membrane viscosity; it is still not clear, however, whether this induces changes in platelet aggregation [1].

In conclusion, we demonstrated that the combination of vitamin E with aspirin reduces the concentration of vitamin E necessary to inhibit platelet aggregation. This might have clinical implications since we used $\alpha$-tocopherol, which is the principal form of vitamin E in human blood [6,12], in a range of concentrations rather close to those of human plasma [6], and theoretically obtainable by means of an appropriate diet. We did not investigate $\gamma$-tocopherol, whose blood concentration is 10-fold lower than that of $\alpha$-tocopherol [6,12], but this needs further study.

The combination of vitamin E and aspirin might represent a new approach when aspirin is not capable of completely inhibiting platelet aggregation [19].

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