

Effects of vitamin E on human platelet and mononuclear cell responses *in vitro*

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Summary. Recent epidemiological studies have provided evidence supporting the potential benefits of antioxidants in coronary prevention. We have investigated the effects of vitamin E on platelets, monocytes and endothelial cells *in vitro*. Pre-incubation of platelets with vitamin E inhibited subsequent thrombin- ($P < 0.05$, $n = 5$), collagen- ($P < 0.0001$, $n = 5$) and ADP- ($P < 0.05$, $n = 4$) induced platelet aggregation measured using a microtitre plate method, or conventional aggregometry. The adhesion of thrombin-activated platelets to collagen was also inhibited by vitamin E ($P < 0.05$, $n = 8$), but not by vitamin C ($P > 0.05$, $n = 8$); nor was the adhesion of unstimulated platelets significantly affected ($P > 0.05$, $n = 8$). Pre-incubation of monocytes with vitamin E inhibited their subsequent adhesion to plastic ($P < 0.05$, $n = 9$), and was also associated with an 18% reduction in adhesion to EA.hy 926 endothelial cells ($n = 8$), although this failed to reach statistical significance. Pre-incubation of the endothelial cells with vitamin E also significantly reduced subsequent mononuclear cell adhesion by 56% ($P < 0.05$, $n = 3$).

Keywords: platelets, mononuclear cells, adhesion, aggregation, vitamin E, α -tocopherol, vitamin C

Vitamin E is a potent fat soluble plasma antioxidant (Burton *et al.* 1983), and it is the predominant antioxidant found in LDL particles. It has been proposed that the oxidative modification of low density lipoprotein (LDL) particles is pivotal in the pathogenesis of atherosclerosis. The 'lipid oxidation hypothesis' of Steinberg *et al.* (1989) has been supported by several recent epidemiological and intervention studies (Rimm *et al.* 1993; Stampfer *et al.* 1993; Stephens *et al.* 1996), and by experimental animal model data (Kita *et al.* 1987).

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Vitamin E, particularly its most biologically active isoform α -tocopherol, is the first antioxidant to be depleted during the oxidation of LDL *in vitro*, indicating its potential importance in preventing LDL oxidation *in vivo*. Epidemiological studies have shown that the plasma levels of vitamin E are low in patients with vascular disease (Gey & Pushka 1989; Riemersma *et al.* 1991), and that its dietary levels are predictive of future coronary events (Rimm *et al.* 1993 Stampfer *et al.* 1993). Secondary prevention studies, using vitamin E supplements have also demonstrated some benefit (Stephens *et al.* 1996). However the precise means by which vitamin E exerts its effects are unclear, and there is evidence that it may be acting by a number of different

mechanisms unrelated to its antioxidant properties (reviewed by Ferns *et al.* 1993). These mechanisms may include potentially antiatherogenic effects on several cell types, including monocytes and platelets (Jandak *et al.* 1989; Salonen *et al.* 1991; Devaraj *et al.* 1996). It is possible that lipophilic compounds, such as vitamin E intercalate into the cell membrane, altering its composition and affecting cell responses. In this current study we have investigated the *in vitro* effects of α -tocopherol on platelet and monocyte function.

Methods

Materials

Eahy cells were a gift from Dr Cora-Jean Edgell, University of North Carolina, USA (Edgell *et al.* 1983). RPMI-1640, Dulbecco's modified Eagle's medium and foetal calf serum were purchased from Life Technologies Ltd. (Scotland, UK). Absolute ethanol was purchased from Romil Ltd (Cambridge, UK). All other reagents were from Sigma Chemical Co. (Dorset, UK).

Platelet isolation

Whole blood was taken from healthy volunteers (mean age 35 ± 12 years) by venesection from an antecubital vein and anticoagulated with 1/10th volume of 3.8% (w/v) trisodium citrate. The blood was centrifuged at room temperature for 20 min at $200 \times g$ and the upper platelet-rich layer harvested. Apyrase (grade 1) and prostacyclin (PGI_2) were added to the platelet-rich plasma at final concentrations of $10 \mu\text{g/ml}$ and $0.33 \mu\text{g/ml}$, respectively. The platelets were pelleted by centrifuging the platelet-rich plasma at $800 \times g$ for 15 min, washed twice in Ca^{2+} -free Tyrode's buffer, and resuspended in Ca^{2+} -free Tyrode's buffer at a concentration of $3 \times 10^{11}/l$.

Platelet aggregometry

Platelet aggregation was assessed by the method of Fratantoni & Poindexter (1990) using an automated temperature-controlled microtitre plate reader (Anthos HTIII, Labtech International, East Sussex, UK), and was independently validated by comparison with conventional analysis using aggregometry. In brief, the time course of platelet aggregation was monitored in flat-bottomed 96-well microtitre plates (Bibby Sterilin Ltd, Staffordshire, UK) by following the change in optical density. Increasing concentrations of thrombin were added to quadruplicate wells at a final concentration ranging from 0 to 2000 units/l. Calcium chloride was

added at to each well at a final concentration of $0.033 \mu\text{mol/l}$. Platelet suspension ($135 \mu\text{l}$) in Ca^{2+} -free Tyrode's buffer at a concentration of $3 \times 10^{11}/l$ was then added to each well, giving a final volume of $150 \mu\text{l}$. The initial absorbance of the wells was measured at a wavelength of 405 nm, and subsequent readings were made at one minute intervals over a period of 20 min. The temperature of the reader was maintained at 37°C throughout the duration of the experiment, and between readings, the plate was shaken at the maximum speed setting. The time taken for the optical density to increase by 0.200 was calculated using Biolize® software (Labtech International, East Sussex, UK). Using this information, a dose-response curve was constructed, and the EC_{50} calculated. The effects of α -tocopherol were examined by preincubating the washed platelets for 1 h at ambient temperature with 1% ethanol (vehicle), or with 100, or 200 $\mu\text{mol/l}$ of α -tocopherol (Sigma Chemical Co., Poole, UK). For standard aggregometry using the method of Born (1962), platelet-rich plasma ($500 \mu\text{l}$) was preincubated for 5 min at 37°C with 1% ethanol, or with 100 or 200 $\mu\text{mol/l}$ of α -tocopherol in a glass cuvette within a Payton aggregometer (Ion Trace Inc., Canada). After the incubation period, the platelet suspension was stirred at a constant rate and agonists were added; collagen (2.5 – $10.0 \mu\text{g/ml}$), or adenosine diphosphate (ADP; 2.5 – $10.0 \mu\text{mol/l}$). The aggregatory response was followed for 5 min after the addition of agonist.

Measurement of platelet adhesion

Washed platelets were prepared as described previously, and the concentration adjusted to $1 \times 10^{11}/l$ in Ca^{2+} -free Tyrode's buffer. Platelet adhesion to tissue culture plastic microtitre plates and collagen coated plates was measured using the method of Bellavite *et al.* (1994). One hundred μl of the platelet suspension was added to uncoated wells and wells coated with collagen (type IV) by incubating with $20 \mu\text{g/ml}$ collagen overnight at 4°C . The platelets were incubated in the wells for 1 h at 37°C . Non-adherent platelets were then removed by washing the plates twice with phosphate-buffered saline using an automated plate washer. After washing the plate, $150 \mu\text{l}$ of 0.1 mol/l citrate buffer (pH 5.4) containing 0.1% (v/v) Triton-X-100 and 5 mmol/l p-nitrophenol phosphate was added, and the plate incubated for 1 h at room temperature. The reaction was terminated by the addition of $50 \mu\text{l}$ of 4 mol/l sodium hydroxide. The absorbance of each well was then measured using an Anthos HTIII plate reader set at a wavelength of 450 nm. The percentage of adherent platelets was calculated using Biolize® software (Labtech

International, East Sussex, UK) with reference to a standard curve of known numbers of platelets prepared for each batch of platelets. The effects of α -tocopherol were examined by preincubating the washed platelets for 1 h at ambient temperature with 1% ethanol (vehicle), or with 100, or 200 $\mu\text{mol/l}$ of α -tocopherol. The effects of concurrent stimulation were assessed by adding thrombin (final concentration 250 U/l), or equivalent volume of Tyrode's buffer to the wells. The effects of ascorbate were assessed by incubating washed platelets with 1 and 2 g/l of ascorbic acid (Sigma Chemical Co., Poole, UK), or an equivalent volume of distilled water. For the latter sample, pH was adjusted to the same value as the preparation containing ascorbic acid by the addition of 5 mol/l citric acid.

Monocyte isolation

Whole blood taken from healthy subjects was anticoagulated with trisodium citrate at a final concentration of 0.38% and centrifuged at $200 \times g$ for 20 min. The platelet-rich supernatant was removed and the remaining packed cell layer restored to its original volume with PBS. Five ml aliquots were overlaid onto Histopaque1077 (Sigma Chemical Co., Poole, UK), and mononuclear cells were prepared according to the method of Byum (1968). In brief the tubes were centrifuged at $400 \times g$ for 30 min, the mononuclear cell layer was recovered and washed twice in 5 volumes of endotoxin-free PBS. Immediately prior to use, the cells were resuspended in serum-free RPMI 1640 medium (Life Technologies Ltd, Scotland, UK) at a concentration of $6 \times 10^9/\text{l}$. Cell viability was assessed by trypan-blue exclusion prior to use, and exceeded 95% on all occasions. The platelet contamination of the mononuclear cell preparation was low (the platelet: mononuclear cell ratio was < 1).

EA-hy 926 endothelial cell line culture

EA-hy 926 cells were a gift from Dr C-J Edgell (University of North Carolina, Chapel Hill, USA). They were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose, 10% fetal calf serum and 100 $\mu\text{mol/l}$ hypoxanthine, 0.4 $\mu\text{mol/l}$ aminopterin and 16 $\mu\text{mol/l}$ thymidine. Cells were grown to confluence for the adhesion assays, and were used at passages 30–40.

Measurement of monocyte adhesion

Aliquots of the suspension of mononuclear cells in serum-free RPMI 1640 medium (6×10^5 cells) were added to 96 well trays containing confluent endothelial cell layers, or cell-free wells. The plates were incubated at 37 °C for 30 min, and nonadherent cells removed by

washing twice with PBS. Monocyte-specific adherence was determined by a modification of the method described by Bath *et al.* (1989). This method relies on the conversion of a colourless substrate, tetramethyl benzidine (TMB) to a blue product by the action of monocyte-specific myeloperoxidase activity. The cells contained in each well were first lysed in 100 μl hexadecyltrimethyl-ammonium bromide (0.5% in PBS; pH 5.0) at 37 °C for 60 min. A freshly made solution of TMB (0.1 mg/ml in 0.05 M phosphate citrate buffer (pH 6.0) containing 0.03% sodium perborate) was added to each well and the plate incubated for 10 min at room temperature. The reaction was stopped by the addition of 50 μl 2.5 mol/l sulphuric acid, and the absorbance measured at 450 nm using an Anthos HTIII microplate reader. A standard curve of cell number vs. absorbance was constructed for each batch of mononuclear cells, and the absolute adhesion calculated by reference to this curve. The effects of α -tocopherol were examined by preincubating the EA-hy-926 cells or mononuclear cells for 1 h at ambient temperature with 1% ethanol (vehicle), or with 100, or 200 $\mu\text{mol/l}$ of α -tocopherol.

Statistical analysis

Statistical analysis was performed using InStat software (Graphpad Inc, USA). Significance was assessed using paired *t*-tests and ANOVA. $P < 0.05$ was considered statistically significant.

Results

Platelet aggregation

Using the modified method of Fratantoni & Poindexter (1990) and the conditions described above, the intra-assay and interassay variabilities for the determination of thrombin EC_{50} were 6.9% and 15.8%, respectively. Preincubation of the washed platelets with α -tocopherol produced a dose-dependent increase in the thrombin EC_{50} ($P < 0.05$, ANOVA, $n = 5$) (Figure 1a), indicating a decrease in platelet sensitivity to thrombin-induced aggregation. Preincubation of platelets with α -tocopherol also inhibited the rate ($P < 0.0001$, ANOVA, $n = 5$) and extent ($P < 0.01$, ANOVA, $n = 5$) of collagen-induced platelet aggregation and the rate of ADP-induced aggregation ($P < 0.05$, ANOVA, $n = 4$) assessed using standard aggregometry (Figure 1b–e).

Platelet adhesion

The intra- and inter-assay coefficients of variability were 5.4% and 9.4%, respectively, for the measurement of

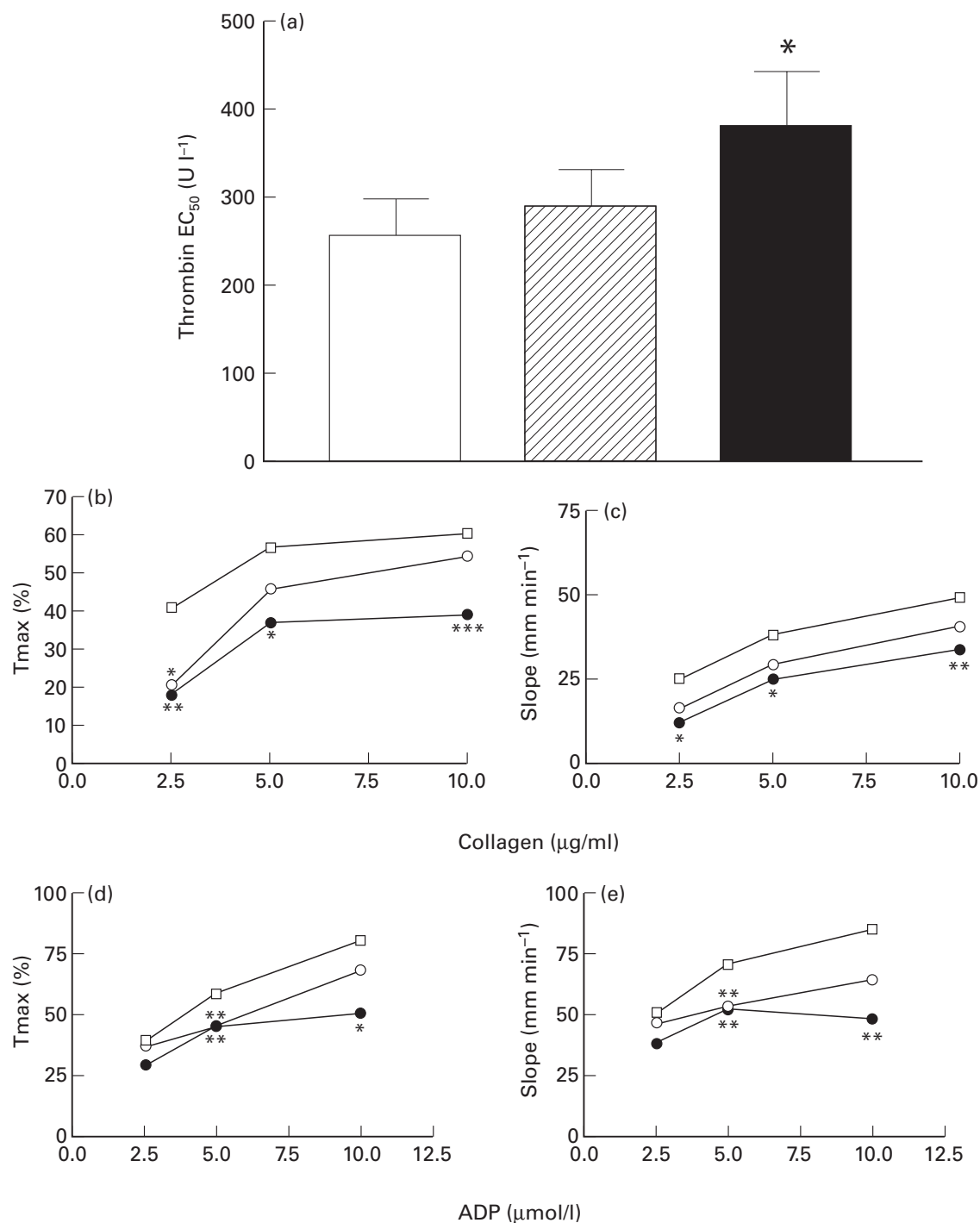


Figure 1. (a) Effect on thrombin-induced aggregation (measured using the microtitre plate technique) of preincubating platelets with α -tocopherol; ▨ 100 μM ; ■ 200 μM ; □ 1% ethanol. Assays were performed in quadruplicate. Data are expressed as the mean \pm SEM ($n=5$ donors). * $P<0.05$ compared to ethanol control. Ethanol was used at a final concentration of 1%, and at this concentration responses did not differ significantly from control samples without ethanol (data not shown). Effects of preincubating platelets with α -tocopherol (○100 $\mu mol/l$; ● 200 $\mu mol/l$) on the extent (T_{max}) and rate (slope) of collagen-(b,c) and ADP-(d, e) induced aggregation using conventional aggregometry. Assays were performed in quadruplicate. Data are expressed as the mean (SEM). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared to ethanol control (□).

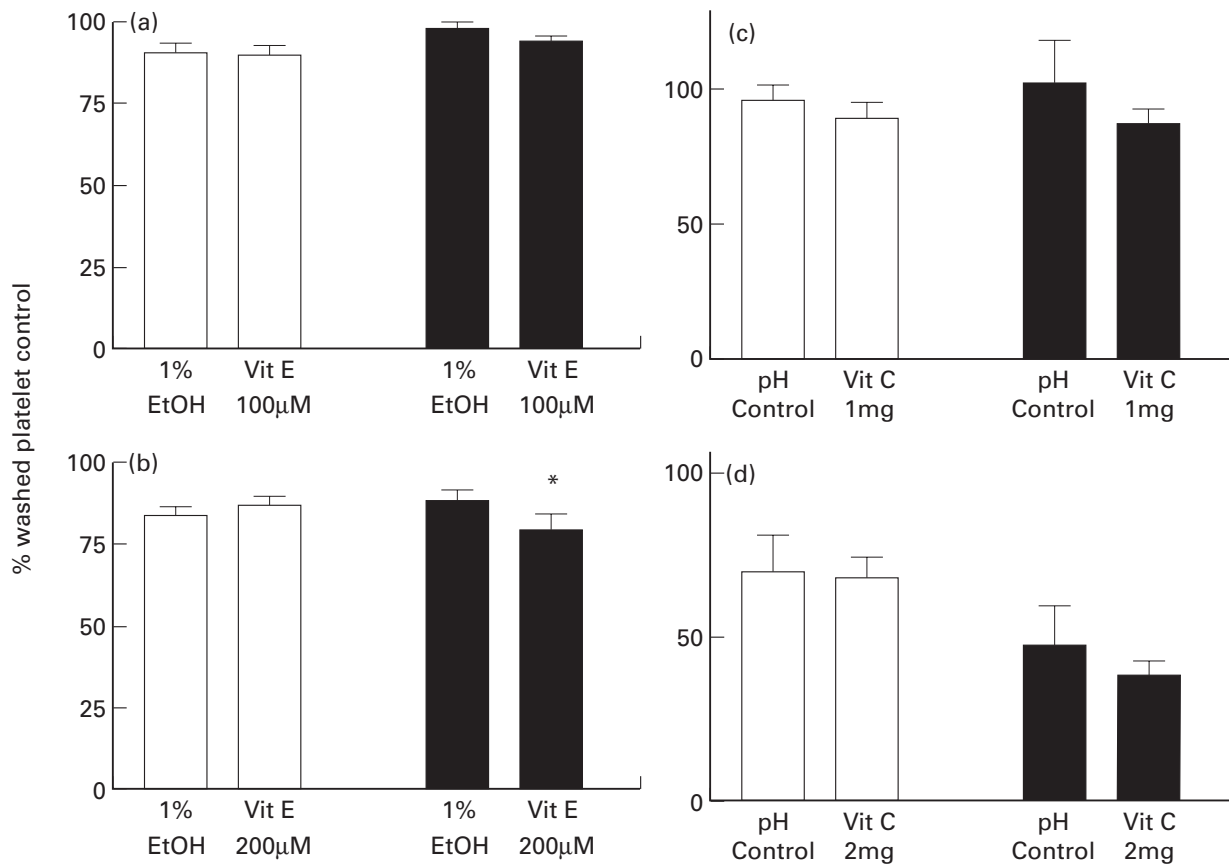


Figure 2. Effects of preincubating platelets with α -tocopherol at (a) 100 μ mol/l and (b) 200 μ mol/l, or ascorbic acid at (c) 1 g/l and (d) 2 g/l on platelet adhesion. The platelets were either in the quiescent state (\square), or stimulated with 250 U/l of thrombin (\blacksquare). Assays were performed in quadruplicate. Values are expressed as the mean (SEM). * $P < 0.05$ compared to ethanol control ($n = 8$ donors).

platelet adhesion using the method of Bellavite *et al.* (1994). The colourimetric assay for platelet acid phosphatase activity was linear up to a concentration of 1×10^{11} platelets/l, and the calibration curve was unaffected by prior activation with thrombin at a concentration of 250 U/l. The adhesion of resting platelets to collagen coated wells was unaffected by preincubation with α -tocopherol at 100 and 200 μ mol/l, however, the adhesion of thrombin-stimulated platelets was inhibited by 10% following preincubation with α -tocopherol at 200 μ mol/l ($P < 0.05$, $n = 8$) (Figure 2a,b). Pre-incubation with 1 and 2 mg with ascorbic acid did not inhibit resting or thrombin-stimulated platelet adhesion (Figure 2c,d).

Monocyte adhesion

The intra- and inter-assay coefficients of variability were 6.7% and 10.4%, respectively, for the measurement of

platelet adhesion using the method of Bath *et al.* (1989). The degree of monocyte adherence was expressed as a percentage of the total number of cells added to wells, and was calculated by reference to a standard curve produced for each individual batch of mononuclear cells. These standard curves were consistently linear up to the maximum number of monocytes added to each well. Pre-incubation of mononuclear cells with α -tocopherol did not significantly inhibit mononuclear cell adhesion to the endothelial cell line, EA.hy 926, although there was an 18% reduction in mean percentage adhesion ($n = 8$). However, the preincubation was associated with a significant inhibition of the adhesion to tissue culture plastic (27% reduction; $P < 0.05$, $n = 9$) (Figure 3a). Pre-incubation of the endothelial cells with α -tocopherol at 200 μ mol/l before the addition of mononuclear cells was also associated with a significant 56% reduction in mononuclear cell adhesion ($P < 0.05$, $n = 3$) (Figure 3b).

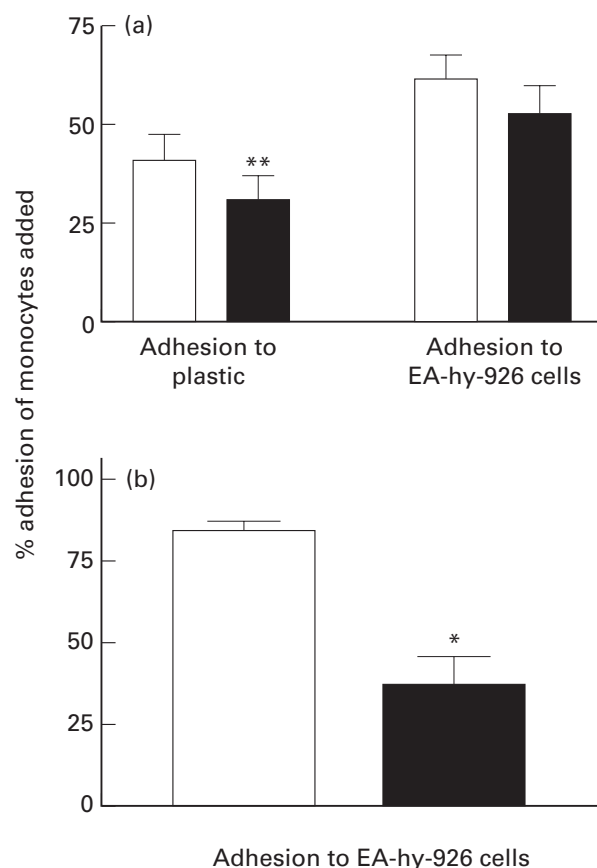


Figure 3. Effects of coincubation of monocytes with α -tocopherol (a) during adhesion to EA.hy 926 endothelial cells ($n=9$ donors) and tissue culture plastic ($n=8$ donors) and (b) preincubating EA.hy 926 endothelial cells with α -tocopherol on mononuclear cell adhesion. ■ 200 μ mol/l; □ ethanol control. Assays were performed in quadruplicate. Values are expressed as mean (SEM). * $P<0.05$ vs. ethanol, ** $P<0.01$ vs. ethanol.

Discussion

Vitamin E inhibits platelet aggregation and adhesion

We have previously shown that dietary vitamin E supplements inhibit thrombin-induced platelet aggregation *ex vivo* in patients with hypercholesterolaemia (Williams *et al.* 1997). Salonen *et al.* (1991) have also shown that dietary supplementation with a cocktail of antioxidants (vitamins C, E β -carotene and selenium) inhibited ADP-induced platelet aggregation. Steiner (1991) could not demonstrate an effect of vitamin E supplements on platelet aggregation at doses of up to 1200 i.u./day, when used in isolation, although platelet adhesion was shown to be inhibited. More recently, Calzada *et al.*

(1997a) have shown that platelets from healthy individuals given vitamin E supplements were less sensitive to ADP, arachidonic acid and collagen-induced aggregation, whereas dietary supplementation with vitamin C and β -carotene had no significant effect. Products of lipoprotein oxidation, such as hydroperoxyeicosatetraenoic acid (HPETE) have been reported to potentiate aggregation (Calzada *et al.* 1997b), and decreased levels of vitamin E in platelets, for example found in patients with diabetes mellitus, was associated with enhanced aggregability (Gerster 1993). Gerster proposed that vitamin E may act by modulating the metabolism of arachidonic acid, or by stabilizing the plasma membrane. Indeed it was found that vitamin E inhibited pseudopodia formation, typically associated with platelet activation. These membrane effects may also be expected to affect platelet adhesion, and Steiner (1993) has reported that vitamin E at doses of 400 iu/day inhibits platelet adhesion to a number of matrices by more than 75%. We found that although vitamin E did not affect the adhesion of resting platelets, the adhesion of thrombin-stimulated platelets to collagen was inhibited by vitamin E, but not by ascorbic acid.

Vitamin E alters monocyte adhesion in vitro

Leucocyte penetration into the arterial wall is also thought to be a key event in atherogenesis. Arterial wall macrophages have the ability to oxidize LDL and are a repository of several potent cytokines and growth factors implicated in atherogenesis. Leucocyte adhesion to the vascular endothelium is the critical first step in this process. Effects of vitamins E and C on monocyte function are also indicated by several *ex vivo* and *in vitro* studies (Ferns *et al.* 1993; Faruqi *et al.* 1994; Devaraj *et al.* 1996; Weber *et al.* 1996). In this present study we were unable to demonstrate a significant reduction in monocyte adhesion to the EA.hy 926 endothelial cell line following preincubation with vitamin E. Monocyte adhesion to plastic was inhibited significantly by vitamin E. This finding may be of some importance, as the adhesion of monocytes to plastic is thought to be mediated by the scavenger receptor, the receptor responsible for the uptake of modified LDL. Pre-incubation of the EA.hy 926 cells with vitamin E also inhibited monocyte binding. We (Ferns *et al.* 1993) have previously shown that dietary antioxidants inhibit monocyte adhesion *in vivo*, a process that appears to be partially mediated by suppression of NF- κ B mobilization within the endothelial cell (Erl *et al.* 1997). Martin *et al.* (1997) have also shown that vitamin E reduces endothelial cell IL-1, prostaglandin I_2 and intercellular

adhesion molecule (ICAM-1) expression following their exposure to LDL.

Conclusions

Pre-incubation of platelets with vitamin E inhibited subsequent thrombin-, collagen- and ADP-induced platelet aggregation. The adhesion of thrombin-activated platelets to collagen was also inhibited by vitamin E, but not by vitamin C; nor was the adhesion of unstimulated platelets significantly affected. Pre-incubation of monocytes with vitamin E inhibited their subsequent adhesion to plastic, and was also associated with a reduction in adhesion to EA.hy 926 endothelial cells, although this failed to reach statistical significance. Pre-incubation of the endothelial cells with vitamin E also significantly reduced subsequent mononuclear cell adhesion. Although the effects of Vitamin E on platelets and mononuclear cells *in vitro* were modest, they are nevertheless likely to be of pathophysiological significance given the relatively short duration of exposure to vitamin E *in vitro*, and the fact that we and colleagues have demonstrated effects of vitamin E *in vivo* following dietary supplementation.

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