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# Effects of Dietary Supplementation with Vitamin E on Human Neutrophil Chemotaxis and Generation of $LTB_4$

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## ABSTRACT

Three weeks' dietary supplementation with a moderate dose of vitamin E (45 IU DL–α-tocopherol acetate daily), in eight healthy volunteers significantly increased the serum vitamin E level from 12.3 ± 3.3 to 16.2 ± 3.7 mg/L (means ± SD) and significantly decreased neutrophil chemotaxis from 15 ± 3 to 4 ± 1 μm/h (means ± standard error of the means). Generation of leukotriene $B_4$ was not influenced by vitamin E, suggesting that the decrease in neutrophil chemotaxis was not due to blockage of the lipoxygenase pathway. Neither was the plasma malondialdehyde concentration influenced by vitamin E, contradicting the possibility of an antioxidant effect of vitamin E. As one early event in neutrophil chemotaxis is an increase in intracellular calcium concentration resulting from increased membrane permeability, it is possible that vitamin E influenced chemotaxis by a stabilizing effect on the neutrophil membrane, rather than by its antioxidant effect. Vitamin E supplementation could thus be beneficial in pathological conditions with activated neutrophils, such as ischaemic heart disease.

## INTRODUCTION

The principal function of vitamin E is to act as a biological lipid antioxidant that scavenges free radical intermediates arising during peroxidation of unsaturated fatty acids (1).

The effects of supplementation of the diet with vitamin E in cardiovascular disease are of interest, as many observations point to the involvement of lipid peroxidation in various stages of the pathological process. Low density lipoproteins (LDL) are readily peroxidized and

taken up by macrophages in atherosclerotic lesions, and peroxidation of LDL is blocked by antioxidants such as vitamin E (20). Further, damaged areas show a lower content of endothelium derived relaxing factor, probably as a consequence of degradation of this mediator by free radicals produced by inflammatory cells in the vessel wall (11). Extremely low vitamin E levels in the plasma have been noted in stroke, and low antioxidant defences have been found in patients with ischaemic heart disease and other angiopathic disorders (8, 12).

Recently it has been suggested that activated leucocytes may play a role in the pathogenesis of ischaemic heart disease, at least partly via release of free radicals and interaction with the endothelium (27).

Neutrophils from rabbits receiving vitamin E have been found to be less adherent than control cells to the endothelial monolayer (14). Also, leucocytes from these rabbits seemed to cause less endothelial cell damage in the pulmonary capillaries. It is thus possible that interference with leucocytes might explain some of the suggested cardioprotective effects of vitamin E. In the present investigation we have therefore examined the effects of a moderate dose of vitamin E on neutrophil chemotaxis and on the production of leukotriene $B_4$ ($LTB_4$) by neutrophils.

## MATERIAL AND METHODS

### *Subjects and Study Design*

Eight healthy volunteers (5 men and 3 women) with a mean age of 49 ± 8 years (mean ± SD, range 33 – 57) were given 45 mg of vitamin E (45 IU DL-α-tocopherol acetate) as tablets once daily for three weeks.

The subjects received no non-steroidal anti-inflammatory or any other drugs or additional vitamin supplements for at least ten days prior to blood sampling. They were told not to change their diets or their lifestyle during the experimental period and not to drink alcohol the day before blood sampling. They fasted overnight and were instructed to avoid vigorous physical activity in the morning of blood sampling, which was always performed at about 8 a.m. The volunteers gave their informed consent. The study was approved by the local Ethical Committee.

### *Blood sampling*

Venous blood samples were taken without stasis with the subjects in the supine position. Samples were taken before and after 3 weeks of dietary supplementation with vitamin E.

*Neutrophil Preparation*

Neutrophils were isolated from heparinized (10 IU/mL) venous blood. The separation was performed by a slightly modified density gradient separation method as previously described (16). To the stock iso-osmotic Percoll (Pharmacia AB, Uppsala, Sweden) was added a solution consisting of one part Hanks' balanced salt solution (HBSS, pH 7.4) with calcium and magnesium and one part 0.075 mol/L Tris-HCl to obtain the desired densities 1.1005 g/mL and 1.0776 g/mL. The blood was layered on this discontinuous Percoll gradient, which was then centrifuged at 400 g for 5 min at 4°C. The platelet-rich plasma supernatant was then removed and centrifugation was continued for 20 min at 800 g at 4°C. The neutrophil band was removed and washed in cold HBSS without calcium and magnesium with 0.1% fatty acid free bovine serum albumin (Sigma, St. Louis, MO), and centrifuged at 400 g for 6 min at 4°C. Ammonium chloride (0.155 mol/L) lysis of the erythrocytes in the cell pellet was used (a cold solution containing 8.29 g/L $NH_4Cl$, 1 g/L $KHCO_3$ and 0.037 g/L $Na_2EDTA$, pH 7.4). The neutrophils were then washed with the same HBSS solution as above and centrifuged at 400 g for 5 min at 4°C. Purified neutrophils were suspended in HBSS with calcium and magnesium for the analysis of $LTB_4$ and in Gey's solution (26) (for random migration) and in Gey's solution with 0.2% human serum albumin (for chemokinesis and chemotaxis). The resulting preparation consistently contained more than 95% neutrophils. The viability was more than 95% as determined by Trypan blue exclusion.

*Neutrophil Chemotaxis*

The migration was assayed by the leading front technique, using a modified Boyden chamber (25). The filter pore size was 3 $\mu$m (Millipore Corp. Bedford, MA) and the filter was about 150 $\mu$m thick. One hundred $\mu$L of neutrophils (final concentration 1.5 x $10^9$/L) was placed above the filter and the chemoattractant, formyl methyl leucyl phenyl alanine (FMLP), was diluted in Gey's solution containing 0.2% human serum albumin and added below the filter. FMLP was prepared as a stock solution in dimethylsulphoxide (DMSO) and kept at –20 °C before use. Neutrophils were allowed to migrate for 60 min at 37°C, whereafter the filters were fixed, stained and mounted. Migration of neutrophils was assayed as the migration distance of the two furthest migrating neutrophils in the focus of one high power field (12.5 x 24) (17). The results are expressed as $\mu$m/h and are the means of readings of three different fields in duplicate filters. Neutrophil chemotaxis was defined as the difference between neutrophil migration in response to FMLP ($10^{-8}$

mol/L, final concentration) and neutrophil migration towards Gey's solution with 0.2% human serum albumin, i.e. chemokinesis or control. Random migration did not change during the experiment.

***Generation of $LTB_4$ by neutrophils***

One millilitre of the neutrophil suspension (5 x $10^9$/L) in HBSS with calcium and magnesium was warmed at 37°C for 2 min and then stimulated with 10 $\mu$mol/L (final concentration) calcium ionophore A 23187. The calcium ionophore was dissolved in DMSO (final concentration 0.5 %) and stored at –70°C before the experiments and then diluted in HBSS. The cell suspension was incubated for 10 min in a shaking water bath and the reaction was stopped by adding 1 mL of ice-cold methanol and placing the tube in ice-cold water. The cell suspension was then centrifuged at 2300 g at 4°C for 15 min and the supernatant was stored at –20°C prior to radio-immunoassay for determination of the generation of $LTB_4$ by neutrophils. The cross-reactivity with $LTB_4$ was 100%, with 20-OH-$LTB_4$ and 6-trans $LTB_4$ 0.4% and with $LTC_4$, $LTD_4$, thromboxane $B_2$ and 6-keto-$PGF_{1\alpha}$ < 0.05%.

***Vitamin E in serum***

The serum was stored at –70°C pending measurement of the vitamin E concentration by high performance liquid chromatography (HPLC) (10). The assays were kindly carried out by Professor Juhani Hakkarainen at the Department of Clinical Nutrition, Swedish University of Agricultural Sciences, Uppsala, Sweden.

***Malondialdehyde in plasma***

Lipid peroxidation in plasma was measured by determining the concentrations of malondialdehyde in plasma by fast performance liquid chromatography (FPLC) with a PEP-RPC™ C–18 reversed phase column after reaction with thiobarbituric acid (22). After blood sampling, 500 $\mu$L of the plasma was immediately mixed with 10 $\mu$L of 5% butylated hydroxytoluene in ethanol and then stored at –70°C until assayed.

***Statistical methods***

Student's *t*-test for paired observations was used to compare values in the same subjects before and after dietary supplementation with vitamin E. Means ± standard deviations (SD) are shown unless otherwise stated; P values < 0.05 were regarded as significant.

## RESULTS

### *Neutrophil Chemotaxis*

A significant decrease in neutrophil chemotaxis, from 15 ± 3 to 4 ± 1 $\mu$m/h, was observed after addition of vitamin E (45 IU daily) to the diet for 3 weeks (means ± standard error of the means; $P < 0.05$; Fig.1).

### *Generation of $LTB_4$ by neutrophils*

The generation of $LTB_4$ by neutrophils did not alter significantly after 3 weeks of dietary supplementation with vitamin E (before treatment: 9.0 ± 1.6 ng/5x$10^6$ cells; after treatment: 9.9 ± 1.1 ng/5x$10^6$ cells; Table 1).

### *Vitamin E in serum*

After dietary supplementation with vitamin E for 3 weeks, the serum vitamin E level was increased to 16.2 ± 3.7 mg/L from an initial value of 12.3 ± 3.3 mg/L ($P < 0.001$; Table 1).

### *Malondialdehyde in plasma*

The concentrations of malondialdehyde in plasma were 0.21 ± 0.05 $\mu$mol/L before the addition of vitamin E to the diet and 0.25 ± 0.09 $\mu$mol/L after 3 weeks of this treatment. This change was not significant (Table 1).

## DISCUSSION

The moderate dose of vitamin E (45 IU daily) given in the diet for three weeks in this study resulted in an increase in the vitamin E level in the serum from 12.3 ± 3.3 to 16.2 ± 3.7 mg/L. Many other investigators have used much higher doses. In one study administration of 1600 IU of vitamin E daily for two or more weeks to volunteers increased the vitamin E level in the serum from about 11 mg/L to about 18 mg/L (3). Although a much lower dose of vitamin E was used in our study, the increase in the serum vitamin E level was almost the same. There is some evidence that absorption of high levels of $\alpha$-tocopherol acetate in humans (>400 IU/d) may be restricted by some limitation in the hydrolysis of the ester at this level of intake (2). It is known from earlier studies that changes in the vitamin E level in leucocytes parallel those in the plasma after dietary supplementation (13).

After addition of vitamin E to the diet for three weeks the neutrophil chemotaxis was significantly decreased. To the best of our knowledge

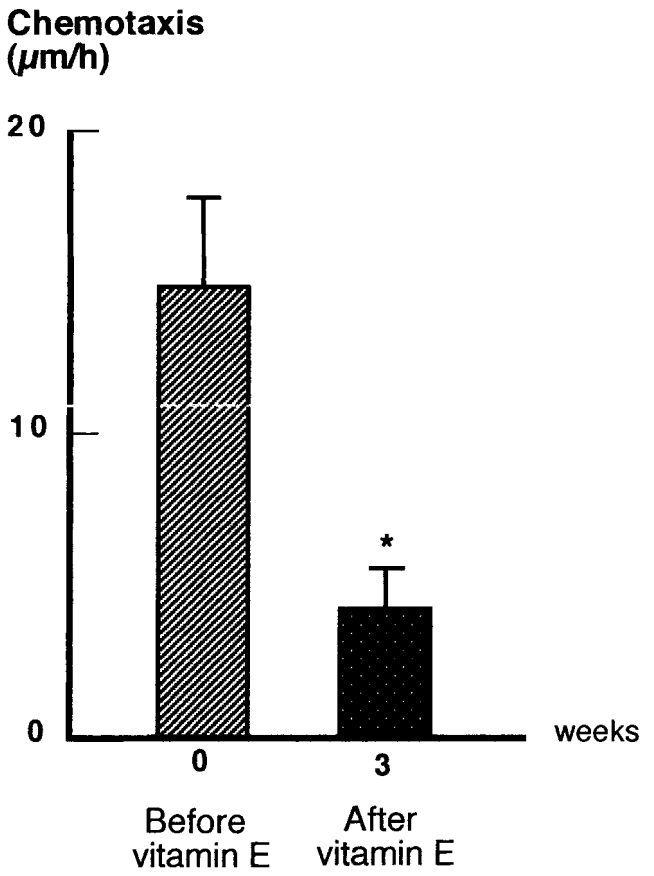


**Figure 1.** The effect of dietary supplementation with vitamin E (45 IU) for 3 weeks on neutrophil chemotaxis (mean ± standard error of the mean, $n$=8, *P<0.05). FMLP, $10^{-8}$ mol/L, was used as a chemoattractant.

such a change in chemotaxis after vitamin E supplementation has not been reported previously. The mechanism of this vitamin E effect is unclear. The generation of $LTB_4$, the 5-lipoxygenase product derived from arachidonic acid, did not alter significantly, suggesting that the change in neutrophil chemotaxis was not due to blockage of the lipoxygenase pathway. Goetzl (9) has shown that physiological levels of α-tocopherol in vitro do not reduce but rather enhance the lipoxygenation of arachidonic acid in human neutrophils. Only higher, unphysiological levels exert a suppressive effect that is consistent with the role of vitamin E as a hydroperoxide scavenger (9). Villa et al. (23) found that vitamin E in vitro inhibited not only arachidonate-induced but also $LTB_4$-induced

**TABLE 1.** The effects of 3 weeks' dietary supplementation with vitamin E (45 IU DL-α-tocopherol acetate daily) on the levels of vitamin E (mg/L, $n$=8) in serum and malondialdehyde (MDA, μmol/L, $n$=8) in plasma and on the generation of $LTB_4$ by neutrophils (ng/5x$10^6$ cells, $n$=7 before and $n$=8 after).

| | VITAMIN E | MDA | $LTB_4$ |
|---|---|---|---|
| **Before** | **12.3 ± 3.3**<br>(8.3 – 16.3) | **0.65 ± 0.05**<br>(0.46 – 0.93) | **9.0 ± 1.6**<br>(6.7 – 11.0) |
| **After** | **16.2 ± 3.7** ***<br>(10.0 – 20.1) | **0.62 ± 0.07**<br>(0.42 – 0.89) | **9.9 ± 1.1**<br>(8.4 – 11.0) |

Values are means and SD, range within parentheses.
***P < 0.001

aggregation of human neutrophils, suggesting that the effects of vitamin E on human leucocyte aggregation may reflect non-lipoxygenase related actions. Vitamin E quinone, an oxidized form of vitamin E, seems to be just as efficient as vitamin E itself in counteracting platelet aggregation (5, 21). These findings make it necessary to look for other explanations than the antioxidant theory as the basic mechanism responsible for the action of vitamin E. Previous studies have shown that vitamin E in vitro exerts antiaggregatory effects in platelets without inhibiting the enzymes of the arachidonic acid cascade, but that the mobilization of intracellular calcium to the cytoplasm is significantly inhibited by vitamin E (4, 5, 19). One of the earliest events in chemotaxis is a rapid increase in the intracellular concentration of calcium, which is a consequence both of calcium displacement from membranous stores and of a selective increase in plasma membrane permeability (18). Vitamin E has also been found to decrease the liposome membrane permeability (6) and it might therefore make the plasma membrane less permeable to calcium. In a recent study it was observed that neutrophils from vitamin E-treated rabbits depolarized more and hyperpolarized more rapidly than placebo cells (24). This finding might indicate differences in the early activation of and ionic flux in neutrophils after administration of vitamin E so that they more rapidly return to a less activated state (24). Vitamin E, which is highly lipophilic,

could thus exert a membrane-stabilizing influence within the membrane lipid bilayer that is not necessarily related to its antioxidant properties.

Recent findings indicate that activated leucocytes might participate in or modify certain pathological conditions, e.g. the development of myocardial infarction, reperfusion arrhythmias or loss of endothelium derived relaxing factor after reperfusion (15). If so, the present results might suggest a role for vitamin E in preventing such conditions by decreasing leucocyte activation. However, the results obtained in the present open study need to be confirmed in a placebo-controlled investigation.

Favouring the possible beneficial effect of vitamin E in cardiovascular disease is the finding that in a current cross-cultural epidemiological investigation of healthy middle-aged men representing 11 European populations, the α-tocopherol/cholesterol ratio in the plasma has shown a strong inverse correlation with mortality from ischaemic heart disease (7).

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