

Vitamin C and E Suppress Mitogen-Stimulated Peripheral Blood Mononuclear Cells in vitro

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Key Words

Vitamin C · Vitamin E · Tryptophan · Neopterin · Kynurenine · Indoleamine-(2,3)-dioxygenase

Abstract

Background/Aims: The antioxidant properties of vitamin C and E are considered to be important for their anti-inflammatory activity. Recently, antioxidant resveratrol was found to suppress neopterin production and tryptophan degradation in mitogen-treated human peripheral blood mononuclear cells. **Methods:** In this study, the effects of vitamin C and E were investigated in unstimulated peripheral blood mononuclear cells and in cells stimulated with the mitogens phytohaemagglutinin and concanavalin A in vitro. **Results:** The mitogens induced a significant production of neopterin and a degradation of tryptophan. Vitamin C (0.1–10 μ M) and E (5–100 μ M) suppressed these immunobiological pathways in a dose-dependent way ($p < 0.01$). **Conclusion:** Neopterin production and tryptophan degradation in monocyte-derived macrophages are both triggered by the pro-inflammatory cytokine interferon- γ . Thus, their concurrent suppression by vitamin C and E suggests an effect on the formation and release of this cytokine by stimulated T cells. These findings may be related to the general health benefits which are associated with the antioxidant nature of these vitamins.

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Introduction

Vitamin C (ascorbate) and E (α -tocopherol) are the most important dietary antioxidants. Vitamin C is water-soluble and exerts its antioxidant properties within the aqueous compartments of blood and cells. It has been described to prevent colds and to improve immune cell function, to promote wound healing, to protect against effects of stress and to be of benefit in cancer prevention [1]. Vitamin E is one of the most potent fat-soluble antioxidants in the body. It protects cell membranes from damage caused by free radicals and, like vitamin C, supports the function of immunocompetent cells, promotes wound healing and reduces the risk of cardiovascular disease and cancer [1, 2]. Likewise, high doses of vitamin E are regarded to prevent low-density lipoprotein oxidation and hence to interrupt the process of atherogenesis [3], and to increase the antioxidative capacity of the liver especially after the ingestion of salmon oil [4].

Various diseases like infections, cancer, autoimmune syndromes as well as coronary heart disease and many other disorders go along with immune activation and oxidative stress. These clinical conditions can be characterized by higher concentrations of immune activation markers like neopterin [5, 6]. Increased amounts of neopterin are produced by human monocyte-derived macrophages and dendritic cells upon stimulation with inter-

feron- γ (IFN- γ) [7, 8]. Parallel to that, IFN- γ stimulates the enzyme indoleamine-(2,3)-dioxygenase (IDO), which converts tryptophan to kynurenine [9]. Accordingly, the ratio of concentrations of kynurenine, the product of IDO, divided by its substrate tryptophan (kynurenine to tryptophan ratio; kyn/trp) allows an estimate of IDO activity [9]. As expected, increased neopterin concentrations in patients usually concur with an enhanced kyn/trp [9].

Also, in vitro neopterin production and tryptophan degradation are induced in parallel for example in peripheral blood mononuclear cells (PBMC) on stimulation with IFN- γ and mitogens like phytohaemagglutinin (PHA) and concanavalin A (Con A) [10]. These mitogen-induced biochemical changes were found to be significantly suppressed by plant extracts and beverages like tea, wine and beer [11–13]. Recently, the pure antioxidant compound resveratrol was found to act in the same way [14]. Thus, the in vitro effects exerted by plant extracts and beverages appear to be related to their high content of antioxidants. To further substantiate this observation, we studied the influence of pure vitamin C and E on stimulated human PBMC in vitro.

Materials and Methods

Test Substances

L(+)-ascorbic acid (176.13 g/mol; Merck, Darmstadt, Germany) and D,L- α -tocopheryl acetate (472.8 g/mol; Neuber GmbH – Brenntag CEE, Vienna, Austria) were applied. After the definition of the dose ranges which were capable of achieving a detectable effect, 3 concentrations of vitamin C (0.1, 1.0 and 10 μ M; mixed 3:1 with anhydrous sodium carbonate in order to achieve neutral pH value) and of vitamin E (5, 50 and 100 μ M) were tested. Three experiments with freshly prepared PBMC of 3 different donors and each with 3 parallel runs were performed with both vitamins.

Isolation of Human PBMC

PBMC were isolated from whole blood obtained from healthy donors. The separation of blood cells was performed using density centrifugation (Lymphoprep; Nycomed Pharma AS, Oslo, Norway). After isolation, PBMC were washed 3 times in phosphate buffered saline containing 0.2% of 0.5 mM EDTA. Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany), 1% of 200 mmol/l glutamine (Serva, Heidelberg, Germany) and 0.1% of 50 mg/ml gentamicin (Bio-Whittaker, Walkersville, Md., USA) in a humidified atmosphere containing 5% CO₂ for 48 h. For each of the experiments, PBMC were freshly prepared. Donors gave informed consent that their donated blood unit may be used for scientific purposes.

Stimulation of PBMC

Isolated PBMC were plated at a density of 1.5×10^6 cells/ml in supplemented RPMI 1640. Cells were preincubated for 30 min with or without the extracts (0.1, 1 and 5%) and exposed or not to mitogens for 48 h. To test for effects of vitamin E, 10 μ g/ml PHA and 10 μ g/ml Con A (both from Sigma, Vienna, Austria) were applied. Because of identical observations made with PHA and Con A in all experiments with vitamin E, solely PHA was tested for studies with vitamin C.

The viability of cells was measured by the trypan exclusion method.

Analytical Procedure

After the incubation of PBMC, supernatants were harvested by centrifugation. Tryptophan and kynurenine concentrations were measured by high-pressure liquid chromatography using 3-nitro-L-tyrosine as internal standard [15]. As an estimate of IDO activity, kyn/trp was calculated and expressed as micromoles of kynurenine per millimole of tryptophan. Neopterin concentrations were determined by ELISA (BRAHMS, Berlin, Germany) according to the manufacturer's instructions with a detection limit of 2 nmol/l. In the supernatants of an additional experiment with vitamin C, IFN- γ concentrations were measured by a commercially available ELISA (Chemikine; Chemicon Europe Ltd., Hampshire, UK).

Statistics

Data are presented as means $\pm$ SEM. To take into account inter-individual differences, the results are presented as n-fold of the unstimulated control cells of each experiment. For the comparison of grouped data, the Mann-Whitney U test was applied. $p < 0.05$ was considered to indicate significant differences.

Results

Unstimulated Cells

The supernatants of unstimulated PBMC contained 28.3 ± 2.4 μ mol/l tryptophan, and by the addition of vitamin C the tryptophan content increased slightly to 31.5 ± 4.2 μ mol/l. Upon the addition of 100 μ mol/l vitamin E, the tryptophan content increased to 32.5 ± 1.0 μ mol/l (data not shown). The baseline kynurenine concentration was 3.2 ± 0.45 μ mol/l and kyn/trp was 112 ± 11.7 μ mol/mmol. The addition of vitamin C led to a slight decrease in kynurenine concentrations and kyn/trp in a dose-dependent manner. Again, vitamin E had similar effects (data not shown).

In the same experiments, an average concentration of neopterin of 9.5 ± 1.2 nmol/l was detected in unstimulated cells. No obvious change of neopterin concentrations was observed upon the addition of vitamin C or E (data not shown).

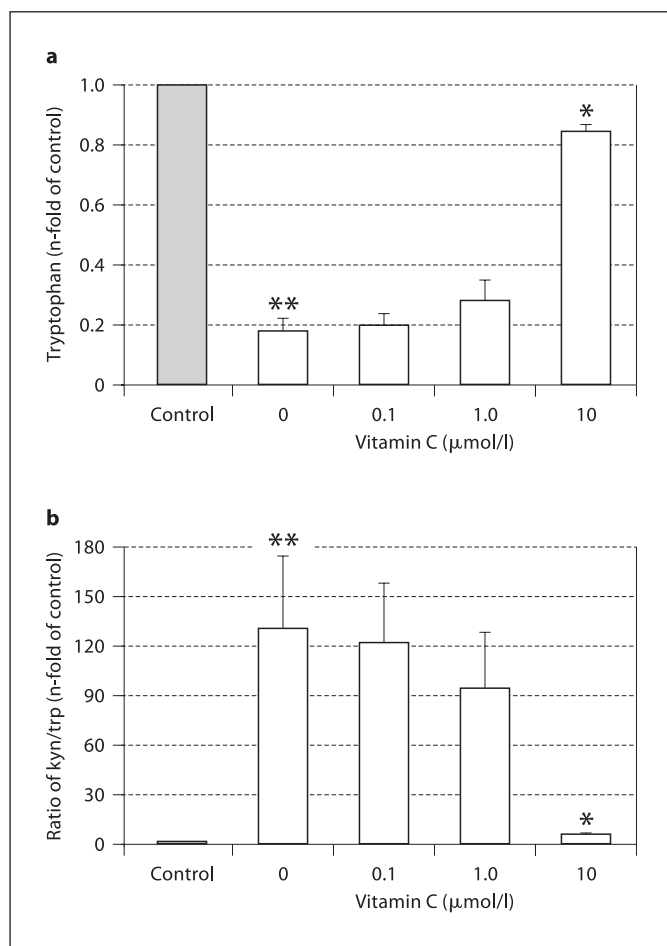


Fig. 1. Concentrations of tryptophan (a) and the ratio of kyn/trp (b) in supernatants of unstimulated PBMC (grey bar, control) and in cells stimulated with PHA (white bars) and treated without (0) or with 3 different concentrations of vitamin C for 48 h. * $p < 0.01$ compared to the stimulated cells without vitamin C (0); ** $p < 0.001$ compared to the unstimulated control cells. Data are presented as n-fold of the control of unstimulated cells.

Mitogen-Stimulated Cells

In PBMC stimulated with the mitogen PHA, a decrease in tryptophan (fig. 1a) and a concurrent increase in kynurenine concentrations were observed (details not shown). The activation of IDO was indicated by a strong increase in kyn/trp (fig. 1b). The addition of vitamin C counteracted the mitogen-induced tryptophan degradation, tryptophan concentrations increased and kyn/trp declined significantly ($p < 0.01$; fig. 1a and b). At a concentration of 10 μM, almost complete inhibition was achieved. At that dose, tryptophan concentrations returned to levels seen in the supernatants of unstimulated

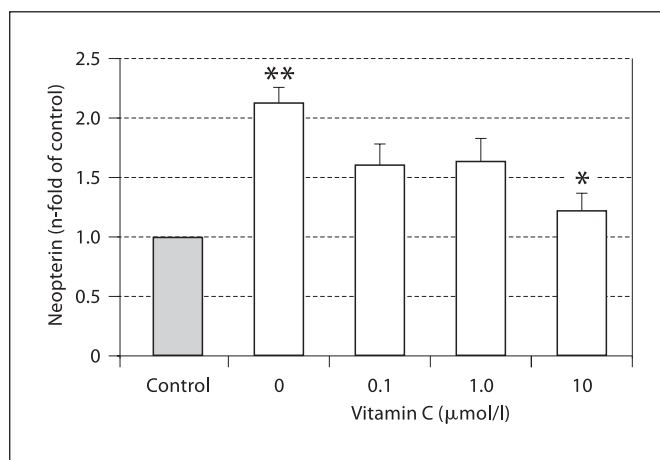


Fig. 2. Concentrations of neopterin in supernatants of unstimulated PBMC (grey bar, control) and in cells stimulated with PHA (white bars) and treated without (0) or with 3 different concentrations of vitamin C for 48 h. * $p < 0.05$ compared to the stimulated cells without vitamin C (0); ** $p < 0.001$ compared to the unstimulated control cells. Data are presented as n-fold of the control of unstimulated cells.

PBMC (28.3 ± 2.4 μmol/l). Kynurenine concentrations tended to reach the level of unstimulated cells at higher concentrations of vitamin C (data not shown).

The stimulation with PHA also strongly induced the neopterin production in PBMC (19.1 ± 3.9 nmol/l; $p < 0.001$ compared to unstimulated controls), and the addition of vitamin C decreased neopterin concentrations (fig. 2). The highest concentration of 10 μM vitamin C significantly lowered neopterin concentrations but it was not high enough to reach the neopterin concentration of unstimulated cells (fig. 2).

The addition of 5–100 μmol/l vitamin E suppressed the mitogen-induced tryptophan degradation and significantly reduced kyn/trp ($p < 0.01$); this was true for either mitogen, i.e. PHA and Con A (fig. 3a and b). Concentrations of 50 and 100 μmol/l of vitamin E almost completely inhibited the mitogen-induced degradation of tryptophan, and the tryptophan concentrations returned to those in the supernatants of unstimulated PBMC. This effect of vitamin E was similar in cells stimulated with PHA, where 31.5 ± 0.8 μmol/l tryptophan was detected, and with Con A, where the level was 31.3 ± 0.7 μmol/l. Parallel to that, the increased neopterin production induced by the mitogens PHA and Con A was suppressed upon the addition of vitamin E in a dose-dependent manner (fig. 4a and b). The addition of 50–100 μmol/l vita-

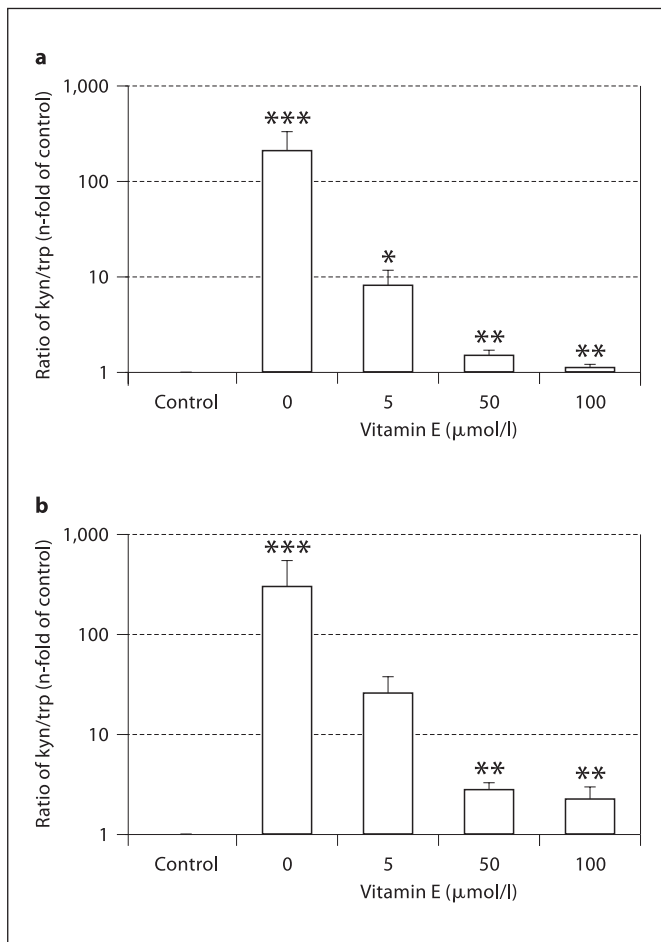


Fig. 3. The ratios of kyn/trp (note log scale!) in supernatants of unstimulated PBMC (grey bar, control) and in cells stimulated with PHA (**a**) or Con A (**b**) and treated without (0) or with 3 different concentrations of vitamin E for 48 h. * $p < 0.05$, ** $p < 0.01$ compared to the stimulated cells without vitamin E (0); *** $p < 0.001$ compared to the unstimulated control cells. Data are presented as n-fold of the control of unstimulated cells.

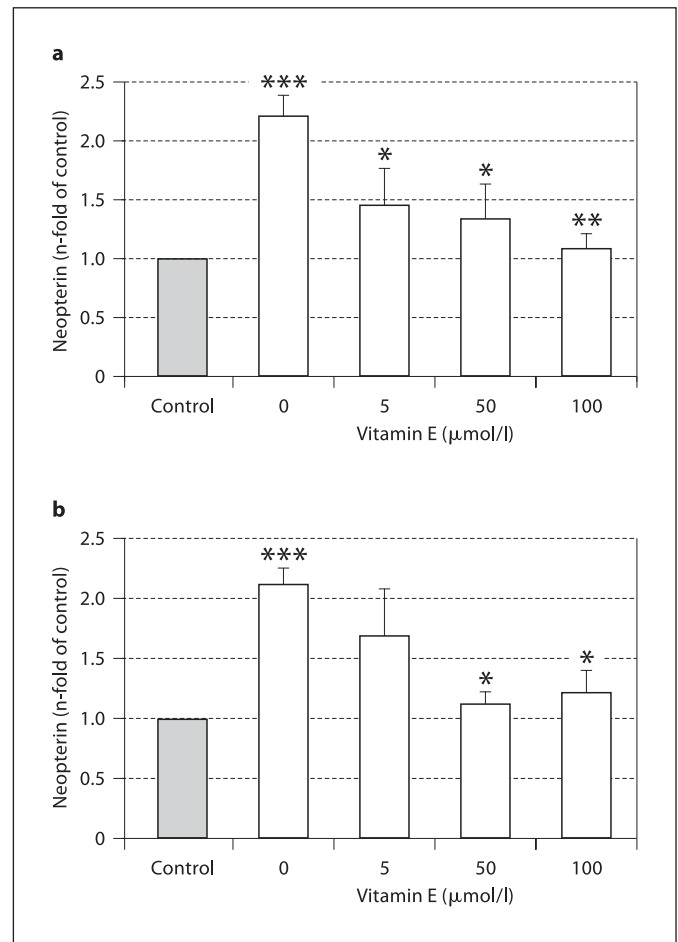


Fig. 4. Neopterin concentrations in supernatants of unstimulated PBMC (grey bar, control) and in cells stimulated with PHA (**a**) or Con A (**b**) and treated without (0) or with 3 different concentrations of vitamin E for 48 h. * $p < 0.05$, ** $p < 0.01$ compared to the stimulated cells without vitamin E (0); *** $p < 0.001$ compared to the unstimulated control cells. Data are presented as n-fold of the control of unstimulated cells.

min E almost completely inhibited any PHA- and Con A-mediated neopterin production.

The stimulation of PBMC with PHA led to an increase in IFN- γ concentrations in the supernatants ($3,586 \pm 29.1$ pg/ml) compared to the unstimulated cells (15.4 ± 1.5 pg/ml; $p < 0.01$). When cells were exposed to vitamin C and E, the decline in PHA-induced IFN- γ production became significant at $10 \mu\text{M}$ vitamin C (563 ± 18.4 pg/ml) and $100 \mu\text{M}$ vitamin E (668 ± 52.8 pg/ml; $p < 0.01$).

The concentrations of vitamin C and E used did not influence the viability of unstimulated and stimulated PBMC.

Discussion

In this study, vitamin C and E were demonstrated to suppress the mitogen-induced neopterin production and tryptophan degradation in PBMC in a dose-dependent manner. Both mitogens, PHA and Con A, very effectively stimulated PBMC, and the suppressive effects of vitamin C and E were similar in cells treated with either mitogen. Both effects, the degradation of tryptophan and the production of neopterin, are triggered by IFN- γ [7, 8, 10], and earlier mitogen-treated PBMC were shown to release significant amounts of IFN- γ , which are related to

neopterin production and tryptophan degradation [16]. Thus, the data of this study are well in line with the conclusion that vitamin C and E suppressed the formation and release of IFN- γ in mitogen-stimulated PBMC. Such results confirm and extend very recent findings on the influence of vitamin C and E on regulatory T cells and dendritic cells in suppressing the release of Th1-type cytokines [17]. However, we cannot exclude the possibility of an additional direct effect of vitamins on neopterin production and tryptophan degradation in monocyte-derived macrophages.

Parallel to enhanced neopterin production and tryptophan degradation, IFN- γ also potently induces the release of reactive oxygen species (ROS) [18]. Therefore, vitamin C and E may not only act as chemical antioxidants by detoxifying ROS [1, 2], they may also reduce the IFN- γ -mediated formation of ROS. In this way, vitamin C and E could have cytoprotective and anti-inflammatory actions. The anti-inflammatory effects may include a generalized reduction in pro-inflammatory mediators and effectors involving, for example, the expression of nuclear factor- κ B [17, 19, 20]. Our data, showing that vitamins suppress mitogen-induced tryptophan degradation and neopterin production in PBMC, are fully in line with this assumption.

Vitamin C and E, like other antioxidant vitamins, have been suggested to limit oxidative damage in humans, thereby lowering the risk of certain chronic diseases [1, 2, 21]. Immune activation and inflammation are deeply involved in the pathogenesis of such diseases in which antioxidant vitamins such as C and E are believed to be of benefit in terms of risk reduction and slowing down disease progression. However, with the only exception of supplemental vitamin E, and possibly vitamin C, being able to significantly lower lipid oxidative damage in both smokers and non-smokers [1], the current evidence is still insufficient to conclude that antioxidant vitamin supplementation reduces oxidative damage in humans [21]. Also, the concentrations effective in our in vitro experiments appear to be rather high, and it is still questionable to what extent one can extrapolate results from in vitro experiments using a high dose of pure vitamins to possible effects in humans. Maybe compounds cannot be resorbed efficiently, and certainly a gradient exists between the concentrations of vitamins present in nutrients, for example, and the ones established in the blood. However, at least in the gastrointestinal tract one can assume the presence of these compounds at reasonable concentrations which may make antioxidant reactions more likely [22]. The antioxidant capacity of ingest-

ed vitamins could be of relevance to establish or shift the redox equilibrium in the gut which could then be of benefit for the whole organism.

By down-regulating Th1-type immunity, vitamin C and E may disturb the balance between Th1- and Th2-type immune response. Thereby, antioxidant vitamins like vitamin C and E could increase the risk for allergic reactions in susceptible patients [23]. In a recent study it was demonstrated that vitamin C and E inhibit nuclear factor- κ B translocation and interrupt oxidative pathways also in human dendritic cells by generating regulatory T cells [17]. Similarly, the vitamin-treated dendritic cells secreted higher levels of Th2 cytokines like interleukin-10, whereas the Th1-type cytokine IFN- γ was decreased. Thus, our data further indicate that excess vitamin C and E may favour Th2-type immune response and thereby may increase the risk of allergy. These findings may help to further understand the basis of allergic responses to citric fruits, which might be particularly relevant when antioxidants like vitamin C are applied as food preservatives in high concentrations (e.g. calcium ascorbate E 302).

In summary, our study demonstrates that vitamin C and E interfere with immunopathogenetic pathways which involve Th1-type cytokine IFN- γ . They seem to interrupt pro-inflammatory cytokine cascades. Our data may relate to the anti-inflammatory property of antioxidant vitamins and to the general health benefits which are associated with their antioxidant nature.

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