Neutrophil superoxide-anion generating capacity in chronic smoking: effect of long-term a-tocopherol therapy

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We investigated whether long-term a-tocopherol therapy in chronic smoking affects superoxide generating capacity of neutrophils *ex vivo*. To this purpose, we randomly assigned 128 male chronic smokers (37 ± 21 pack years of smoking) to treatment with placebo ($n = 64$) or a-tocopherol (400 IU dL-a-tocopherol daily, $n = 64$). After two years of therapy, we measured phorbol 12-myristate 13-acetate-induced superoxide production of isolated neutrophils and of diluted whole blood by monitoring reduction of ferricytochrome c and luminol-enhanced peroxidase-catalyzed chemiluminescence. Plasma lipids and lipoproteins were not different between the two treatment groups. As expected, concentrations of a-tocopherol in plasma and in low-density lipoproteins were markedly elevated in the supplemented group compared to the placebo group ($+ 120\%$, $P < 0.0001$ and $+ 83\%$, $P < 0.0001$, respectively). Consequently, resistance to *in vitro* oxidation of low-density lipoproteins (reflected by lag time of conjugated diene formation) was higher in the supplemented group than in the placebo group ($+ 22\%$, $P < 0.0001$). Superoxide generating capacity of neutrophils and superoxide production in diluted whole blood did not differ between a-tocopherol and placebo group. It is concluded that in chronic smoking long-term supranormal a-tocopherol intake does not reduce neutrophil superoxide-anion generating capacity, despite large increases in the concentrations of a-tocopherol in plasma and in low-density lipoproteins.

1. Introduction

Apart from the ability to scavenge reactive oxygen species (ROS) during lipid peroxidation, the lipophilic antioxidant a-tocopherol has been shown to influence cellular functions (Azzi *et al* 2002). The mechanism underlying these activities of a-tocopherol involves inhibition of protein kinase C (PKC) (Mahoney and Azzi 1988) and prevention of activation of intracellular redox-sensitive signal transduction pathways such as nuclear transcription factor kappa B (NF*k*B) (Meyer *et al* 1994). Of special interest are those cellular functions that are directly related to conditions of oxidative stress, such as production of ROS by phagocytic cells. *In vitro* respiratory burst of rat peritoneal neutrophils could be inhibited by addition of a-tocopherol to the cell suspension (Kanno *et al* 1995). That this effect of a-tocopherol was not confined to the test-tube was shown in two other studies revealing an inhibitory effect of a-tocopherol on superoxide production by monocytes and neutrophils *ex vivo* after supplementation of a-tocopherol to human subjects. Firstly, Devaraj *et al* (1996) have shown decreased release of ROS by monocytes *ex vivo* in response to lipopolysaccharide (LPS) after a-tocopherol supplementa-

tion. Secondly, in non-smoking human subjects we have recently shown decreased production of superoxide by isolated circulating neutrophils *ex vivo* after short-term treatment with *a*-tocopherol (van Tits *et al* 2000). Moreover, in that study, assessment of superoxide production using the diluted whole blood assay yielded similar data. A decreased superoxide generating capacity of phagocytic cells may reduce detrimental effects of ROS to lipids, proteins and DNA (Klaunig *et al* 1997). This may be relevant, for instance, in atherosclerosis where oxidation of low-density lipoprotein (LDL) plays a key role, and neutrophils, the predominant phagocytes in the circulation, have been suggested to mediate this early step. We therefore investigated whether a two-years *a*-tocopherol therapy in chronic smoking affects superoxide generating capacity of circulating neutrophils cells *ex vivo*.

2. Materials and methods

2.1 *Subjects*

Subjects were male cigarette smokers (59.9 ± 6.0 years, $n = 128$) who had smoked an average of 42 ± 7 years, all recruited from the general population. None of the subjects suffered from diabetes mellitus or current illness interfering with participation. Subjects did not use antilipidemic drugs, vitamin K antagonists, or (multi) vitamin, vitamin E, vitamin C, *b*-carotene, garlic, or fish oil supplements. The study was designed as a two-years randomized double-blind placebo-controlled trial. Smokers received either capsules containing a daily dose of 400 IU (268 mg) vitamin E as dL-*a*-tocopherol ($n = 64$) or placebo capsules ($n = 64$), provided by Hoffman La Roche Ltd (Basel, Switzerland). Fasting blood samples for analysis of different parameters, were collected before and after the intervention. Written informed consent was obtained from all participants, and the protocol of the study was approved by the ethics committees of University Medical Center Nijmegen and Wageningen University.

2.2 *Isolation of polymorphonuclear leukocytes*

Heparinized whole blood was layered onto Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) within 30 min after withdrawal and centrifugated according to the method of Boyum (1968). Polymorphonuclear (PMN) cells were isolated from the pellet fraction of Ficoll-Paque gradient by lysis of the erythrocytes in 155 mmol/l NH_4Cl/10 mmol/l $KHCO_3$ buffer (Kuijpers *et al* 1991), washed twice in PBS and suspended in Hank's Balanced Salt Solution (HBSS, Gibco Life Technologies, Paisley, Scotland) supplemented with 0·25% human albumin

(HSA, Behringwerke AG, Marburg, Germany). PMN suspensions had a purity of > 99%.

2.3 *Superoxide production*

About two hours after withdrawal of blood, production of superoxide by isolated PMN in response to phorbol 12-myristate 13-acetate (PMA; 50 ng/ml; Sigma-Aldrich) was assessed as reduction of ferricytochrome *c* (Kessels *et al* 1993) and by monitoring luminol-enhanced peroxidase-catalyzed chemiluminescence (CL) (van Tits *et al* 2000). Maximum rate of reduction of cytochrome *c* was determined at 37ºC at 550 nm on a thermostatted Perkin-Elmer Lambda 12 spectrometer (Perkin-Elmer, Norwalk, CT, USA) and expressed in nmol/min per 10^6 PMN, using 21·1 mmol/l/cm as the extinction coefficient for (reduced-oxidized) cytochrome *c* (Van Gelder and Slater 1962). Due to technical reasons, reduction of cytochrome *c* was not performed for all subjects (placebo, $n = 55$; *a*-tocopherol, $n = 53$). Peak CL-activity of PMN was measured on a Victor 1420 multilabel counter (Wallac, Turku, Finland) at room temperature using white 96-well microplates (catalogue No. 3096; Corning Costar Corporation, Cambridge, MA, USA). Each well contained 2×10^5 cells, 50 µM luminol (Sigma-Aldrich), and 4·5 U/ml horseradish peroxidase (hrp; Sigma-Aldrich) in 200 µl HBSS/HSA 0·25%. Integrated CL-activity of heparinized whole blood (final dilution 3000 × in HBSS/HSA 0·25%) during 20 min following PMA stimulation (50 ng/ml) was measured identically within one hour after blood withdrawal in a microplate luminometer MicroLumat Plus LB96V (Berthold Co., Wildbad, Germany) as described earlier (van Tits *et al* 2000). Hrp was included in the CL assay in order to overcome peroxidase deficiency extracellularly (Lundqvist and Dahlgren 1996). In previous experiments we observed that the addition of extra peroxidase did not affect superoxide production – measured as reduction of cytochrome *c* – of PMN stimulated with PMA, but increased luminol-enhanced CL 3- to 4-fold. Hence, it is solely the detection of superoxide that is enhanced in the presence of extra peroxidase.

PMA-induced reduction of cytochrome *c* by PMN and PMA-induced CL activity of PMN and of whole blood could be totally quenched with superoxide dismutase (50 U/ml, final concentration) and prevented with the PKC inhibitor staurosporine (50 nM, final concentration), indicating the exclusive reflection of superoxide production and the specificity of these assays, respectively (data not shown). The methods were specific for cellular superoxide production since in the absence of cells no superoxide production could be detected.

Since measurement of cellular activities over a two-year interval is difficult to standardize, production of

superoxide was measured at the end of the intervention period. To exclude analytical variations, equal numbers of placebo-treated and **a**-tocopherol-treated subjects were assayed at the same time.

2.4   *Other methods*

Cholesterol and triglyceride concentrations in serum were determined by enzymatic methods (Boehringer-Mannheim, Mannheim, Germany) on a Hitachi 747 analyzer (Hitachi, Tokyo, Japan). Concentrations of **a**-tocopherol were analysed by reversed phase high-performance liquid chromatography (de Graaf *et al* 1991). Susceptibility of LDL, isolated from EDTA plasma supplemented with saccharose before storage at – 80ºC, to *in vitro* oxidation, was monitored by the change in absorbance at 234 nm as described by Esterbauer *et al* (1989) and as modified by Princen *et al* (1992). Lag time, defined as the time interval between the intercept of the linear least-square slope of the absorbance curve with the initial-absorbance axis, was taken as a measure of resistance to oxidation.

2.5   *Data analysis and statistics*

The computer program ASTUTE (Microsoft Ink, Redmond, WA, USA) was used for the analysis. Data are presented as means ± SDs and evaluated by using Student's *t*-test for unpaired data. Linear regression analysis and Pearson correlation test were performed to examine the relationship between variables.

3.   Results

Plasma lipids and lipoproteins of placebo and **a**-tocopherol treated smokers are presented in table 1. No differences in lipids and lipoproteins were observed between the two groups. As expected, concentrations of **a**-tocopherol in plasma and in LDL were markedly elevated in the supplemented group of smokers compared to the placebo group (+ 120%, $P < 0.0001$, and + 83%, $P < 0.0001$, respectively; figure 1). Resistance to *in vitro* oxidation of LDL, as reflected by lagtime of conjugated diene formation, was also higher in the supplemented group than in the placebo group (+ 22%, $P < 0.0001$; figure 1).

Production of superoxide by PMN and in whole blood in response to PMA-stimulation did not differ between **a**-tocopherol and placebo group (figure 2). Spontaneous production of superoxide amounted less than 0·5% of PMA-stimulated values and was not different between groups (data not shown). Similar rates of PMA-stimulated superoxide production by human PMN were found by others (Kessels *et al* 1993). A significant positive correlation between peak CL activity and maximum rate of reduction of ferricytochrome *c* by stimulated PMN was observed (figure 3). Integrated CL activity during 20 min following stimulation of whole blood with PMA was weakly associated with maximum rate of reduction of ferricytochrome *c* by PMN ($r = 0.28$, $P = 0.003$, $n = 108$).

4.   Discussion

In the present study, we assessed superoxide production capacity of phagocytic cells of chronic smokers supplemented daily either with 400 IU dL-**a**-tocopherol or with placebo for two years. Measurements were performed both in isolated PMN preparations and in diluted whole blood, and we used two different methods to specifically detect superoxide: monitoring of reduction of ferricytochrome *c*, and luminol-enhanced peroxidase-catalyzed CL. Although these are totally different methods and they were performed at different temperatures, a significant positive correlation was observed between peak CL activity and maximum rate of reduction of ferricytochrome *c* by isolated PMN. In the whole blood assay, not a maximal response but the integrated CL activity during 20 min post stimulation was measured. Therefore, this parameter only weakly correlated with maximum rate of reduction of ferricytochrome *c* by isolated PMN. Spontaneous generation of superoxide by PMN was low, indicating minimal activation due to the isolation procedure. We found no difference in *ex vivo* production of superoxide by PMN of smokers treated either with placebo or **a**-tocopherol. Recently, we did observe a decreased capacity of PMN to generate superoxide in response to PMA

Table 1.   Plasma lipids and lipoproteins of smokers after intervention.

	Placebo treated ($n = 64$)	**a**-tocopherol treated ($n = 64$)	*P*-value
Plasma total cholesterol (mmol/l)	5·85 ± 0·79	5·98 ± 0·95	0·40
Triglycerides (mmol/l)	1·58 ± 0·60	1·78 ± 0·91	0·14
HDL cholesterol (mmol/l)	1·20 ± 0·30	1·15 ± 0·33	0·37
LDL cholesterol (mmol/l)	3·99 ± 0·82	4·08 ± 0·86	0·53

after short-term supplementation of *a*-tocopherol (6 weeks, 600 IU/day) to normolipidemic and hypertriglyceridemic subjects (van Tits *et al* 2000). Concentration of *a*-tocopherol in plasma and LDL of these subjects had increased 2- to 3-fold. In the present study similar increases in plasma and LDL *a*-tocopherol concentrations were observed. Devaraj *et al* (1996) have previously shown

that such increases in plasma and LDL *a*-tocopherol concentrations are accompanied by a comparable increase in monocyte *a*-tocopherol content. Thus, it may be expected that leukocyte *a*-tocopherol content of subjects supplemented with *a*-tocopherol in the present study has also markedly increased. Possibly, during the two-year supplementation period, cellular physiology has adapted
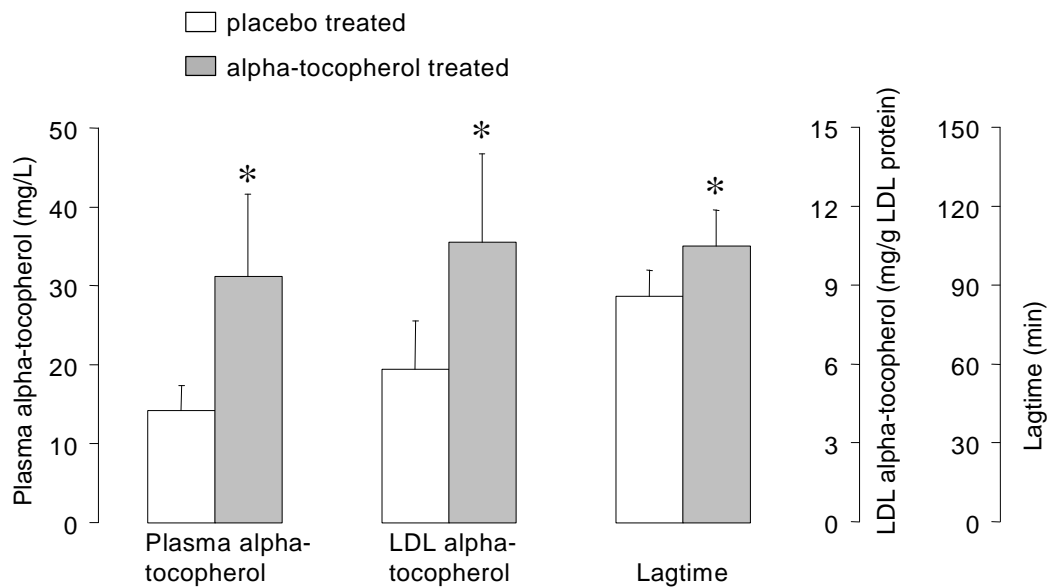

Figure 1. Concentrations of *a*-tocopherol in plasma and in LDL and lagtime of *in vitro* LDL oxidation of smokers treated with placebo or *a*-tocopherol. *, $P < 0.0001$ (*t*-test versus placebo treated).
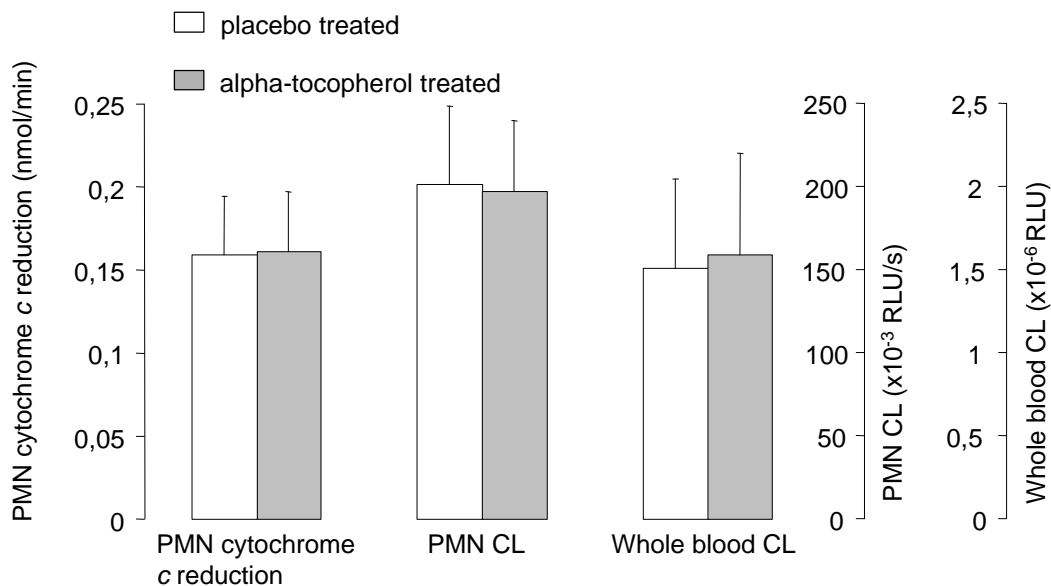

Figure 2. Production of superoxide by polymorphonuclear cells (PMN) and in whole blood of smokers treated with placebo or *a*-tocopherol. CL, chemiluminescence.
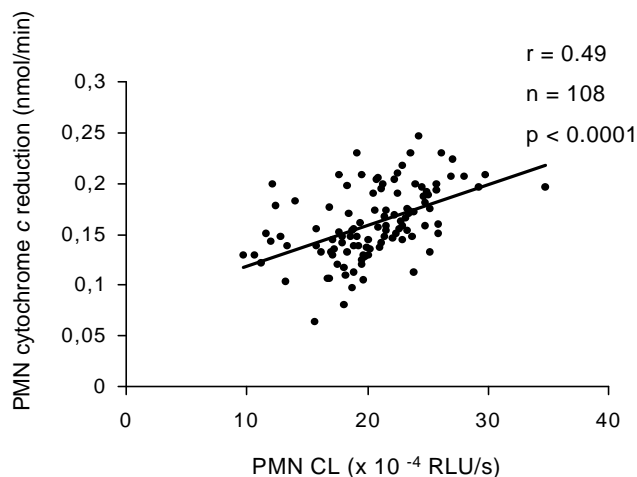
Figure 3. Superoxide production by PMN cells stimulated with phorbol 12-myristate 13-acetate: correlation between peak CL activity and maximum rate of reduction of ferricytochrome *c*. RLU, Relative light units.

to the elevated *a*-tocopherol content, thus preventing changes in cellular functions.

To our knowledge, this is the first study investigating long-term effects of *a*-tocopherol on cellular functions. We did not perform paired measurements (pre and post intervention), but compared measurements done on placebo- and *a*-tocopherol-treated well-matched subjects. Groups were sufficiently large to detect small changes in superoxide production. In contrast to short-term treatment we found no effect of long-term treatment with *a*-tocopherol on respiratory burst of isolated circulating neutrophils and of whole blood. On the other hand, the antioxidant effect of *a*-tocopherol was present since resistance to *in vitro* oxidation of LDL was significantly higher in *a*-tocopherol-supplemented subjects. Whether other functions of these circulating leukocytes, or functions of other cells such as lung neutrophils and macrophages (which may be more important with respect to bacterial infections) are affected, remains to be investigated.

In conclusion, long-term supplementation of *a*-tocopherol to chronic smokers has a direct antioxidant effect but does not additionally inhibit superoxide production by neutrophils, and thus may not reduce LDL oxidation during oxidative stress.

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