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α-Tocopherol increases caspase-3 up-regulation and apoptosis by β-carotene cleavage products in human neutrophils

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Abstract

It has been found that β-carotene cleavage products (CarCP), besides having mutagenic and toxic effects on mitochondria due to their prooxidative properties, also initiate spontaneous apoptosis of human neutrophils. Therefore, it was expected that antioxidants such as α-tocopherol would inhibit the stimulation of apoptosis and caspase-3 activity by CarCP. However, we found that α-tocopherol increases caspase-3 up-regulation and stimulation of apoptosis of human neutrophils by CarCP. Ascorbic acid does not alter this caspase-3 up-regulating and proapoptotic effect exerted by α-tocopherol. Both α-tocopherol and ascorbic acid, in the absence of CarCP, decrease intracellular caspase-3 activity and spontaneous apoptosis of neutrophils. Uric acid alone or in combination with CarCP does not exert apparent effects on caspase-3 activity and apoptosis. Up-regulating effect of α-tocopherol is not observed in the presence of retinol that markedly stimulates apoptosis by itself, whereas increase of caspase-3 activity is induced by concomitant addition of α-tocopherol and β-ionone, a cyclohexenyl degradation product of β-carotene with shorter aliphatic chain.
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1. Introduction

Epidemiological studies indicated that high consumption of fruits and vegetables rich in carotenoids is associated with low risk for various forms of cancer, cardiovascular disease and some age-related degenerative diseases [1]. However intervention trials showed little, no or even detrimental effects of β-carotene supplementation on human health [2]. An increase in the risk of lung cancer among smokers and asbestos workers who were supplemented by high dosage of β-carotene was also reported [3–5].

Prooxidative effect of β-carotene cleavage products was discussed as possible reason for the procarcinogenic action in the case of malignant lesions [6,7]. It was reported that carotenoids do behave as oxygen "carriers" from peroxyl radicals to cellular targets [8]. A switch from prooxidant to antioxidant behaviour might be achieved at relatively low oxygen partial tension as well as by adding vitamin E and C [8]. Synergistic protection against peroxynitrite and nitrogen dioxide radicals was observed in mixture of β-carotene, vitamin E and C, as compared to the individual anti-oxidants, and this was explained in terms of an electron transfer in which the β-carotene radical is repaired by vitamin C [9].

We recently reported that carotenoid derivatives induce apoptosis of human neutrophils and suggested that premature neutrophil death may greatly increase the potential risk for host tissue injury [10]. In this report, we studied the effect of β-tocopherol and ascorbic acid on the apoptosis of human neutrophils induced by carotenoid cleavage products. The scavenger effect of uric acid, an inhibitor of peroxynitrite-related chemical reactions [11], was also studied.

2. Materials and methods

All chemicals were from Sigma (St. Louis, MO) unless stated otherwise and were of the highest grade of purity available. Urate stock solution was prepared

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by dissolving uric acid in 1 M NaOH; the solution was neutralized by addition of HCl before use. Stock solutions of β-tocopherol and benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD; Calbiochem-Behring, San Diego, CA) were prepared in methanol (MetOH) and dimethyl sulfoxide (DMSO), respectively. Ascorbic acid was dissolved in water immediately before the experiments.

Mixtures of carotene cleavage products (CarCP) were prepared by mixing 40 μM methanolic solution of β-carotene (supplied by Cognis Pty Limited, Sydney, Australia) with 1 mM hypochlorous acid at room temperature as previously described [12]. When the "bleaching" reaction was finished, as indicated by a stable colour in the reaction mixture, the cleavage products were extracted twice in hexane. The hexane phases were combined and then evaporated and dried with nitrogen. The residues were redissolved in aliquots of DMSO, which were stored at −80 °C until used. Degradation of 10 μmol β-carotene yielded 5.5 mg of dried cleavage products.

Blank solutions without carotene cleavage products, β-tocopherol, ascorbic acid and uric acid were prepared under exactly the same conditions employed to dissolve these compounds in stock solutions. The solutions were used as blank controls for the experiments with the cells.

Leukocytes were purified from heparinized human blood freshly drawn from healthy donors. Leukocyte preparations containing 90% to 98% neutrophils and apparently free of contaminating erythrocytes were obtained by one-step procedure involving centrifugation of blood samples layered on Ficoll–Hypaque medium (Polymorphprep, Axis-Shield, Oslo, Norway) [13]. The cells were suspended in isotonic phosphate-buffered saline (PBS), pH 7.4, with 5 mM glucose and stored in ice. Each preparation produced cells with a viability higher than 90% up to 6 h after purification. The viability of the cells was measured by trypan blue exclusion test. The incubations were carried out at 37 °C.

Caspase-3 activity was tested in neutrophil lysates by measuring the release of the fluorescent 7-amino-4-methyl-coumarin (AMC) moiety from the synthetic substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AcDEVD-AMC). Neutrophils (5×10^6 cells) were collected by centrifugation and lysed in 0.5 ml volumes of 50 mM HEPES, 5 mM 3-[3-(cholamidopropyl) dimethylammonio]-1-proanesulfonate (CHAPS), 5 mM dithiothreitol (DTT), pH 7.4. The reaction was started by adding 100 μl aliquots of the lysates in 2 ml solutions containing 16 μM AcDEVD-AMC, 20 mM HEPES, 0.1% CHAPS, 5 mM DTT, 2 mM EDTA, pH 7.4. The assay mixtures were incubated at 20 °C in the dark for 1–1.5 h. The fluorescence increase was compared with solutions containing appropriate blank controls, 10 μM acetyl-Asp-Glu-Val-Asp-al, a specific caspase-3 inhibitor [14], or standard preparations of recombinant caspase-3. A calibration curve obtained with standard AMC solutions was employed for quantitative analysis. The excitation and emission wavelengths of AMC were 360 and 460 nm, respectively.

Fragmentation of neutrophil DNA was tested by agarose gel electrophoresis. Neutrophils (5×10^6 cells) were incubated for 20 min in 400 μl of ice-cold hypotonic buffer (10 mM Tris–HCl, 10 mM EDTA, 0.2% Triton X-100, pH 8.0). After addition of 100 μl NaCl (5 M) and 500 μl isopropanol, the samples were held at −20 °C for 60 min and then centrifuged at 14,000 rpm for 10 min. The dried pellet was resuspended in 30 μl of a buffer containing 90 mM Tris–borate, 25 mM EDTA, pH 8.3. The fragments of DNA (1 μg per lane) were separated by electrophoresis in 1% agarose gel and stained with ethidium bromide.

Specific differences among treatments were examined using Student's *t* test that assumed unequal variations.

3. Results

Apoptosis was assessed by measuring caspase-3 activity in lysates of neutrophils (5×10^6 cells/ml) that had been pre-incubated for 3.5 h at 37 °C. If the cells were suspended in phosphate buffered saline with 5 mM glucose, caspase-3 activity in the cell extracts was roughly equivalent to the production of 2 pmol AMC/(min × 10^6 cells).

Addition of carotenoid cleavage products (13 μg/ml, corresponding to 24 μM β-carotene) in 0.28 M DMSO caused a two-fold increase of intracellular caspase-3 activity in the neutrophil suspension (Table 1), in good agreement with previously reported results [10]. The proapoptotic effect exerted by the carotenoid cleavage products was less pronounced but still evident in the presence of 0.25 M MetOH. Both DMSO and MetOH did not significantly affect intracellular caspase-3 activity in the absence of carotenoid cleavage products (Table 1).

α-Tocopherol alone (10, 20, 50, or 100 μM in 0.25 M MetOH) caused a slight but statistically significant decrease of intracellular caspase-3 activity ($P<0.05$), while a pronounced up-regulation of intracellular caspase-3 activity was observed for combinations of various concentrations of α-tocopherol and carotenoid cleavage products (13 μg/ml). The effect of the combined treatment (see Fig. 1, second to fifth column pairs) was considerably higher than that observed in the presence of carotenoid derivatives alone, which is presented by the first column pair from the left side of Fig. 1.

Uric acid (50, 100, 250, or 500 μM), used alone or in combination with carotenoid cleavage products, did not exert apparent effect on the intracellular caspase-3 activity (Fig. 2). Ascorbic acid (100 μM) slightly decreased intracellular caspase-3 activity ($P<0.05$), if used alone, but did not alter significantly the caspase-3 up-regulating and proapoptotic effect exerted by α-tocopherol in combination with carotenoid cleavage products (Fig. 3).

Retinol (10–20 μM) and β-ionone (20 μM), two degradation products of β-carotene that still retain cyclohexenyl ring, exerted different effect on neutrophil caspase-3 activity (Fig. 4). Retinol stimulated caspase-3 activity in a concentration-dependent manner, but its proapoptotic effect was mitigated by α-tocopherol. β-Ionone did not significantly affect the enzyme activity, if used alone, but stimulated caspase-3 activity in combination with α-tocopherol ($P<0.05$).

Chromatin fragmentation was examined as a qualitative marker for neutrophil apoptosis (Fig. 5). If neutrophils were preincubated for 5 h at 37 °C in PBS, gel electrophoresis demonstrated a slightly increased amount of low-molecular-weight DNA. In the presence of 13 μg/ml carotenoid cleavage products, neutrophils exhibited low-molecular-weight DNA in larger quantities, which were electrophoresed in a dense ladder pattern. This effect was more evident by using 13 μg/ml carotenoid cleavage products in combination with α-tocopherol, in particular with 100 μM α-tocopherol. Similar results

Table 1
Effect of carotenoid cleavage product and solvents on the intracellular caspase-3 activity

	caspase-3 activity pmol/(min × 10^6 cells), mean ± SD	
	none	13 μg/ml CarCP
None	2.2 ± 0.3	
0.28 M DMSO	1.9 ± 0.1	4.1 ± 0.1 (*)
0.28 M DMSO ± 0.25 M MetOH	2.2 ± 0.1	3.2 ± 0.1 (*)

Intracellular caspase-3 activity was determined as described in the text. Values are given as mean ± SD ($n=4$). Significant difference (*$P<0.05$) is observed versus solvent alone.

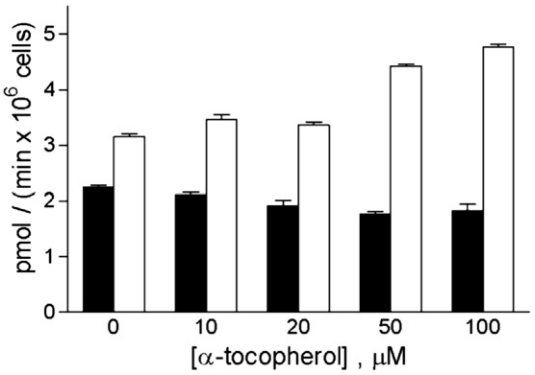


Fig. 1. Combined effect of tocopherol and carotenoid cleavage products on neutrophil caspase-3 activity. The purified cells were suspended in PBS containing 5 mM glucose, 0.28 M DMSO and 0.25 M MetOH in the absence (closed bars) and presence (open bars) of 13 μg/ml carotenoid cleavage products and 0–100 μM α-tocopherol. Cell suspensions were incubated at 37 °C for 3.5 h. Intracellular caspase-3 activity was determined as described in the text. Values are given as mean+SD (n=4). Significant difference (P<0.05) is observed versus controls without α-tocopherol.

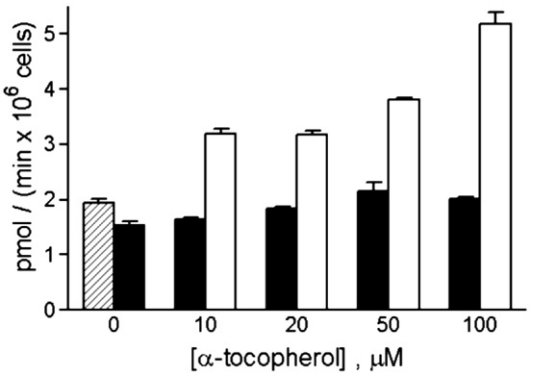


Fig. 3. Combined effect of α-tocopherol, ascorbic acid and carotenoid cleavage products on neutrophil caspase-3 activity. The purified cells were suspended in PBS containing 5 mM glucose, 0.28 mM DMSO, 0.25 M MetOH and 100 μM ascorbic acid in the absence (closed bars) and presence (open bars) of 13 μg/ml carotenoid cleavage products and 0–100 μM α-tocopherol. Shaded bar refers to an experiment performed in absence of vitamin derivatives. Cell suspensions were incubated at 37 °C for 3.5 h. Intracellular caspase-3 activity was determined as described in the text. Values are given as mean±SD (n=4). Significant difference (P<0.05) is observed versus controls without α-tocopherol.

were obtained by incubating neutrophils in the presence of 0.5 mM cycloheximide. Control experiments showed, furthermore, that chromatin fragmentation by carotenoid cleavage products and α-tocopherol was greatly reduced in the presence of *z*-VAD, a broad-spectrum caspase inhibitor [15].

4. Discussion

This report shows that carotenoid cleavage products induce apoptosis in human neutrophils and that the concomitant addition of α-tocopherol enhances the apoptotic effect of the carotenoid cleavage products including caspase-3 up-regulation. The proapoptotic activity of carotenoid cleavage products was affected neither by uric acid, an efficient antioxidant that has recently emerged as peroxynitrite scavenger [11,16], nor by ascorbic acid that has been reported to exert, in the presence of β-carotene and α-tocopherol, synergistic protective effect from oxidative stress in model systems [9]. By the contrast, in the absence of carotenoid cleavage products, α-tocopherol and ascorbic acid slightly decreased caspase-3 activity and neutrophil apoptosis, while uric acid did not exert significant effect. Previous works [10] indicated that superoxide production was very low under the employed experimental conditions, since we used fresh preparation of neutrophils in resting state and a relatively high carotenoid derivative concentration (corresponding to 24 μM α-carotene), which inhibits the already very low production of oxygen radicals by unstimulated cells.

The results are in agreement with recent findings of Miyoshi et al. (2005) who demonstrated an α-tocopherol-mediated

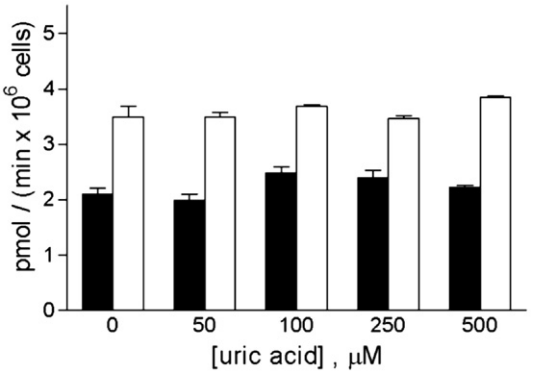


Fig. 2. Combined effect of uric acid and carotenoid cleavage products on neutrophil caspase-3 activity. The purified cells were suspended in PBS containing 5 mM glucose and 0.28 M DMSO in the absence (closed bars) and presence (open bars) of 13 μg/ml carotenoid cleavage products and 0–500 μM uric acid. Cells suspensions were incubated at 37 °C for 3.5 h. Intracellular caspase-3 activity was determined as described in the text. Values are given as mean±SD (n=4).

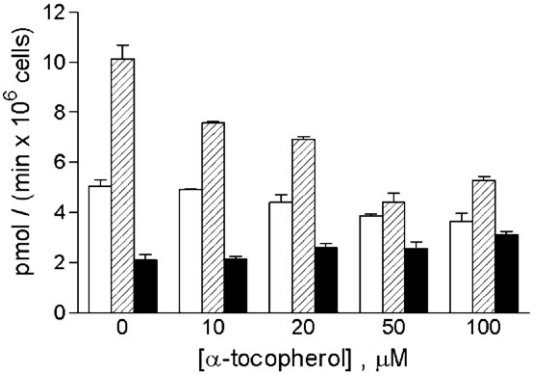


Fig. 4. Combined effect of tocopherol, retinol and β-ionone on neutrophil caspase-3 activity. The purified cells were suspended in PBS containing 5 mM glucose and 0.25 M MetOH in the presence of retinol (open bars: 10 μM; shaded bars: 20 μM), 20 μM β-ionone (closed bars) and 0–100 μM α-tocopherol. Cell suspensions were incubated at 37 °C for 3.5 h. Intracellular caspase-3 activity was determined as described in the text. Values are given as mean±SD (n=4). Significant difference (P<0.05) is observed versus controls without α-tocopherol.

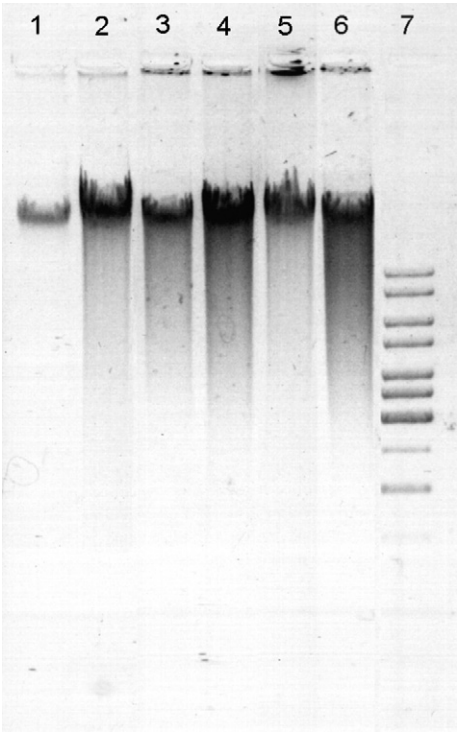


Fig. 5. Chromatin fragmentation in neutrophils exposed to carotenoid cleavage products, tocopherol and z-VAD. Agarose gel electrophoresis patterns observed with molecular size markers (line 7), neutrophils incubated at 0 °C for 5 h in absence of effectors (line 1), neutrophils incubated at 37 °C for 5 h in absence of effectors (line 2), neutrophils incubated at 37 °C for 5 h in presence of 13 μg/ml carotenoid cleavage products (line 3), 13 μg/ml carotenoid cleavage products and 100 μM α-tocopherol (line 4), 13 μg/ml carotenoid cleavage products, 100 μM α-tocopherol and 20 μM z-VAD (line 5), 0.5 mM cycloheximide (line 6). Other experimental conditions are reported in the text.

caspase-3 up-regulation enhancing the susceptibility to apoptotic stimuli such as Fas, hydrogen peroxide, or etoposide in several human cell lines [17]. In those experiments, α-tocopherol also elevated the pro-caspase-3 protein level and mRNA expression in a time- and dose-dependent manner. α-Tocopherol promoted caspase-3 expression without changing the specific activity of the enzyme.

The results question the role of carotenoid breakdown products, which are known to exert toxic effect on mitochondria and even mutagenic effects [7,18,19], as intermediates in redox reactions in human neutrophil apoptosis. The findings are somewhat unexpected since several independent observations support a role for oxidative mechanisms in this process [20,21]. It has been suggested that the detrimental effect of carotenoids is due to the prooxidative properties of β-carotene and of its oxidation products [8,9]. The formation of carotenoid radical cations has been regarded as an intermediate step in the reaction cascade triggered by peroxyl radicals that are produced by the cells and damage the cell structures [22,23]. A relationship between apoptosis induction and changes of intracellular redox potential has been observed in human adenocarcinoma cells [24]. Indeed, it has been reported that, unlike human neutrophils, adenocarcinoma cells increase oxygen radical production in the presence of high concentrations of β-carotene. β-Carotene induced apoptosis in adenocarcinoma cells and both the prooxidant and the growth-inhibitory effects of β-carotene were reversed by addition of the antioxidant α-tocopherol in these cells.

Although the prooxidative and proapoptotic effects of carotenoids are well documented, the pathways for induction of apoptosis are still controversial. It has recently reported [25] that β-tocopherol did not protect T-lymphoblast cell line from apoptosis induced by carotenoids. Moreover, the antioxidant BO-653 led to a slight but statistically significant increase of cell apoptosis when it was used alone, and to a dramatic increase of apoptosis when it was used in combination with β-carotene. It was suggested that a second pathway by which carotenoids might induce apoptosis is via the formation of retinoic acid or retinoic acid analogues [26,27]. Other mechanisms, which were taken into account to explain cell apoptosis, include direct DNA damage [27] and down-regulation of cyclooxygenase-2 activity [28] and epidermal growth-factor receptors [29]. The cooperative apoptotic effect of the antioxidant BO-653 in combination with β-carotene is still unexplained. Here we have, with α-tocopherol, an antioxidant, which is able to stimulate caspase-3 up-regulation and apoptosis of human neutrophils induced by a mixture of β-carotene cleavage products. Up-regulating effect of α-tocopherol was not observed in the presence of retinol that markedly stimulated apoptosis by itself, whereas increase of caspase-3 activity was induced by concomitant addition of α-tocopherol and β-ionone, a cyclohexenyl breakdown product of β-carotene with shorter aliphatic chain. It is noteworthy to mention that the effect of carotenoid cleavage products on neutrophil viability was partially influenced by the addition of methanol. This suggests that solvents, which may influence the repartition of lipophilic vitamins between different compartments in the cells, should be considered to explain, at least in part, the effects exerted by carotenoid derivatives in combination with other compounds.

Because of the obvious difficulty to compare in vitro with in vivo models, caution must be employed in discussing the physiological role of cooperative proapoptotic effect of α-tocopherol and carotenoid cleavage products. Anyhow, it must be stressed that the concentrations of α-tocopherol and carotenoid derivatives employed in our work are comparable with those present in human tissues. It is well known [30] that α-tocopherol, independently of its antioxidant scavenging ability, affects signal transduction and gene expression in different tissues and inhibits cell proliferation. The recommended optimal plasma concentration of α-tocopherol is 30 μM or greater, which corresponds to a daily intake of about 15–30 mg of vitamin E [31,32]. Plasma concentration of β-carotene is highly variable and depends on the biological availability of this compound in food [33]. β-Carotene levels in patient's serum were 3 μM after the intake of 24 mg β-carotene per day in an observation of Stahl et al. [34] and 4 μM in the CARET study [3]. High concentrations of carotenoid cleavage products in combination with excessively high number of neutrophils are present very likely in situations of heavy oxidative stress in various organs such as lung (smoking, environmental pollution, ozone, inflammation) [35–37] and in sun- or photoirradiated

skin after local application of carotenoid containing cosmetics [38,39].

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