The carotenoid β-cryptoxanthin stimulates the repair of DNA oxidation damage in addition to acting as an antioxidant in human cells

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The role of dietary antioxidants in human health remains controversial. Fruits and vegetables in the diet are associated with lower rates of chronic disease, and this is often attributed to their content of antioxidants, and a resulting protection against oxidative stress. However, large-scale human trials with antioxidant supplements have shown, if anything, an increase in mortality. We have investigated the biological properties of β-cryptoxanthin, a common carotenoid, in cell culture model systems, using the comet assay to measure DNA damage. At low concentrations, close to those found in plasma, β-cryptoxanthin does not itself cause damage, but protects transformed human cells (HeLa and Caco-2) from damage induced by H2O2 or by visible light in the presence of a photosensitizer. In addition, it has a striking effect on DNA repair, measured in different ways. Incubation of H2O2-treated cells with β-cryptoxanthin led to a doubling of the rate of rejoining of strand breaks and had a similar effect on the rate of removal of oxidized purines by base excision repair. The latter effect was confirmed with an in vitro assay: cells were incubated with or without β-cryptoxanthin before preparing an extract, which was then incubated with substrate DNA containing 8-oxo-7,8-dihydroguanine; incision was more rapid with the extract prepared from carotenoid-preincubated cells. No significant increases were seen in protein content of human 8-oxoguanine DNA glycosylase 1 or apurinic endonuclease 1. The apparent cancer-preventive effects of dietary carotenoids may depend on the enhancement of DNA repair as well as antioxidant protection against damage.

Introduction

Diet certainly plays an important role in preventing cancer, but the mechanism is still not clear. Epidemiological evidence links consumption of fruits and/or vegetables with decreased risk of cancer (1).

Reactive oxygen species produced in cells, largely as by-products of metabolic processes, constantly threaten the integrity and correct functioning of cellular DNA (2). Several oxidant species have the capacity to produce promutagenic lesions in DNA (3), which may play a significant role in the development of cancer. Fruits and vegetables contain relatively high amounts of components with inherent antioxidant properties, such as vitamin C, carotenoids and flavonoids, which has led to the suggestion that dietary antioxidants may augment cellular antioxidant defences and help protect cellular components from oxidative damage. Antioxidant activity is therefore considered to play an important role in the protective effects of fruits and vegetables against cancer (4,5).

β-Cryptoxanthin, one of the six major carotenoids routinely measured in human serum (β-carotene, lycopene, lutein, β-cryptoxanthin, zeaxanthin and α-carotene), is obtained primarily from citrus fruits (6), but is also found in corn, peas and some yellow coloured animal products such as egg yolk and butter. Like other carotenoids, β-cryptoxanthin is an antioxidant and may help prevent free radical damage to biomolecules including lipids, proteins and nucleic acids. Retinoids, the cleavage products of carotenoids, as well as being antioxidants, have in some cases (including β-cryptoxanthin) vitamin A activity and may play an important role in the prevention and treatment of certain cancers (7,8).

There are several prospective studies investigating the possible effect of β-cryptoxanthin (and other carotenoids) on risk of various cancers. Toniolo et al. (9) found that levels of β-cryptoxanthin and lutein in serum were linked with reduced risk of breast cancer in a dose-dependent fashion. A similar negative association of breast cancer risk with plasma carotenoid levels was reported by Tamimi et al. (10). β-Carotene, lycopene, β-cryptoxanthin, zeaxanthin and lutein are reduced in colorectal adenomas, suggesting that mucosal carotenoids could serve as biomarkers for predisposition to colorectal cancer (11). Women with high circulating concentrations of β-cryptoxanthin and tocopherol may be at a reduced risk of cervical atypical squamous cells of undetermined significance (12). However, Dorgan et al. (13) found a positive association between several plasma carotenoids, including β-cryptoxanthin, and risk of squamous cell carcinoma in a 5 year follow-up trial of patients who had already had a non-melanoma skin cancer.

Supplementation trials with carotenoids, aimed at reducing risk of cancer, have had disappointing results. A recent meta-analysis (14) showed, if anything, an increase in cancer risk following supplementation with β-carotene, vitamin A and vitamin E, singly or in combination. Thus, carotenoids in general must be regarded with circumspection, and further investigations of their modes of action are warranted.

Here, we use the comet assay (alkaline single-cell gel electrophoresis) to investigate the ability of β-cryptoxanthin to protect against DNA oxidation damage in cultured Caco-2 and HeLa cells and also the possible enhancement of DNA repair (a second line of defence). The comet assay is a simple, fast and reliable method for measuring DNA strand breaks (SBs). A simple modification, incorporating digestion of DNA with lesion-specific enzymes, endonuclease III and formamidopyrimidine DNA glycosylase (FPG), allows measurement of oxidized pyrimidines and oxidized purines, respectively (15). DNA repair can be studied with the comet assay in different ways. If cells are subjected to damage (strand breakage or base oxidation) and then incubated to allow them to repair, the lesions remaining can be measured at intervals. Alternatively, an in vitro approach can be used, in which a cell extract is incubated with lysed agaro-embedded substrate cells containing specific damage. The ability of the extract to introduce DNA breaks in the substrate cells reflects the activity of the enzymes responsible for the initial steps of repair, i.e. removing the lesion. The assay was tested on Ogg1+ and wild-type mouse fibroblast cell lines (16): there was essentially no incision activity in the extract from the mutant cells, confirming that it measures principally 8-oxoguanine DNA glycosylase 1 (OGG1) enzyme activity.

Materials and methods

Cell culture

HeLa cells (derived from human cervical cancer) and Caco-2 cells (derived from human colon carcinoma) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were maintained as monolayer cultures at 37°C in a humidified atmosphere with 5% CO2 and routinely passaged by trypsinization when nearly confluent.
Comet assay

Cells were trypsinized and resuspended in phosphate-buffered saline (PBS) at 10⁶ cells/ml. Thirty microlitre of cell suspension was mixed with 140 μl of 1% low-melting point agarose, and two 70 μl aliquots were dropped onto a microscope slide precoated with 1% normal melting point agarose. Glass cover slips were placed on the drops of agarose, which were left to set at 4°C. The cover slips were removed and the agarose-embedded cells were simply lysed. All slides were then placed in a cold alkaline electrophoresis solution (0.3 M NaOH and 1 mM Na₂EDTA, pH 13). Electrophoresis was carried out in this solution for 30 min at 25 V and 30°C. The slides were washed in cold 0.4 M Triton X buffer (pH 7.5) for 10 min to neutralize excess alkali and for 10 min in water and left to dry overnight.

Gels were stained with 25 μl of 4',6-diamidino-2-phenylindole (1 μg/ml), covered with a cover slip and coded. Using a fluorescence microscope, 100 comets (50 on each gel) were visually classified into five categories, 0–4, representing increasing levels of DNA damage.

Preparation of cell pellets and extracts.

HeLa and Caco-2 cells were treated with 40 μl of M Ro plus 0.25% Triton X-100 and reaction buffer was used, and as a positive control FPG. After incubation, alkaline unwinding and electrophoresis proceeded as described in the standard comet assay. Three independent experiments were performed using extracts from different cell cultures.

Western blotting

Whole-cell extract. Aliquots of 10⁶ HeLa and Caco-2 cells, untreated or after incubation for 2 h with 4 μM β-cryptoxanthin, were centrifuged and snap frozen as described above. Pellets were resuspended in 1 ml of cold radioimmunoprecipitation assay buffer [0.15 M NaCl, 25 mM Tris, 1% NP40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 2 mM dithiothreitol, 1× proteinase inhibitor cocktail (Sigma-Aldrich, St Louis, MO), pH 7.5] and left on ice for 10 min. The suspension was sonicated and centrifuged at 16 000g for 20 min at 4°C to remove cell debris.

Nuclear extract. Frozen pellets were resuspended in an equal volume of cold nuclear extraction buffer 1 (10 mM KCl, 10 mM HEPES, 1.5 mM MgCl₂, 1 mM dithiothreitol and 1% proteinase inhibitor cocktail, pH 8.0) and left on ice for 15 min. The suspension was then forced four times through a 23-gauge needle, centrifuged at 16 000g for 20 s and the pellet resuspended in two-third extraction buffer plus 0.25% Triton X-100 and dilute with reaction buffer following the same procedure as the one used to make extracts from cell pellets. The incubation was performed exactly as with the extracts.

To a negative control (0 μl of a mixture of extraction buffer plus 0.25% Triton X-100 and reaction buffer was used, and as a positive control FPG. After incubation, alkaline unwinding and electrophoresis proceeded as described in the standard comet assay. Three independent experiments were performed using extracts from different cell cultures.

Stimulation of DNA repair by β-cryptoxanthin

β-cryptoxanthin on endogenous and induced DNA damage

Stock solutions of β-cryptoxanthin (Extrasthesyn, Lyon, France) for all experiments were prepared in dimethyl sulphoxide (DMSO) in the dark. The final concentration of DMSO in the culture medium was <1%. HeLa and Caco-2 cells were incubated with 0, 1, 4, 10 or 25 μM β-cryptoxanthin for 1 h at 37°C in the dark. The comet assay was performed to assess the effect of β-cryptoxanthin on endogenous DNA SSBs and—with the inclusion of FPG digestion—the effect on oxidized purines. To examine the ability of β-cryptoxanthin to protect against induced oxidative damage, preincubated cells were washed with PBS and treated on ice with H₂O₂ (25 μM for HeLa cells and 15 μM for Caco-2 cells) for 5 min to induce SSBs or with 1 μM Ro (photostimulator 1890-8022, from F.Hoffmann-La Roche, Basel, Switzerland) plus visible light from a 500 W tungsten-halogen source (2.5 min for HeLa cells and 1.5 min for Caco-2 cells) at 33 cm to induce 8-oxoGua. The comet assay was performed without or with FPG to measure SSBs or 8-oxoGua, respectively. Three independent experiments were performed.

Cellular repair assays

Rejoining of SSBs was followed after treating cells with H₂O₂ and repair of oxidized bases after treating them with Ro plus light. In each case, three independent experiments were performed.

HeLa and Caco-2 cells were treated with H₂O₂ (30 μM for HeLa cells and 20 μM for Caco-2 cells) for 5 min on ice to induce SSBs. H₂O₂ was washed off with PBS and cells were incubated with 1 or 4 μM β-cryptoxanthin for 0, 5, 15, 30 and 45 min for HeLa cells and 0, 15, 30, 45 min and 1 h for Caco-2 cells at 37°C in the dark. To follow repair of oxidized bases, cells were treated with 1 μM Ro plus light (2.5 min for HeLa cells and 1.5 min for Caco-2 cells) to induce oxidized bases. Ro was washed off with PBS and cells were incubated with 1, 4, 10 or 25 μM β-cryptoxanthin for 0, 0.5, 1, 2 and 4 h for HeLa cells and 0, 1, 2, 4 and 8 h for Caco-2 cells at 37°C in the dark.

Measuring in vitro repair

This in vitro assay (16) provides a measure of the incision activity of a cell extract provided with a DNA substrate from cells containing specific damage—8-oxoGua. These cells are embedded in agarose, lysed and left to dry overnight. A pellet was thawed and resuspended in 33 μl of extraction buffer (45 mM HEPES, 0.4 M KCl, 1 mM ethylenediaminetetra-acetic acid, 0.1 mM dithiothreitol and 10% glycerol, pH 7.8) to which Triton X-100 was added to 0.25% just before use. Vortex mixing for 5 s at top speed was followed by incubation for 5 min on ice and centrifugation at 14 000g for 5 min. Twenty-eight microlitre of supernatant was removed and combined with 110 μl of cold reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM ethylenediaminetetra-acetic acid and 0.2 mg/ml bovine serum albumin, pH 8).

The reaction. Pairs of gels containing ~2 × 10⁶ substrate HeLa cells, with or without treatment with Ro plus light, in 70 μl of 1% low-melting point agarose in PBS were set on agarose-precipitated glass slides, and cells were lysed for 1 h as in the standard comet assay (see above). Slides were washed for 3 × 5 min with reaction buffer. Thirty microlitre of extract was placed on each gel, covered with a cover slip and incubated for 10 min in a humid chamber at 37°C. To check whether β-cryptoxanthin present in the extract directly induces DNA breaks, substrate membranes from damaged cells were treated with β-cryptoxanthin. In this case, β-cryptoxanthin in 10 μM DMSO at concentrations of 0.1, 0.4, 1 and 4 μM was mixed with extraction buffer plus 0.25% Triton X-100 and then diluted with reaction buffer following the same procedure as the one used to make extracts from cell pellets. The incubation was performed exactly as with the extracts.

As a negative control (0 μl of a mixture of extraction buffer plus 0.25% Triton X-100 and reaction buffer was used, and as a positive control FPG. After incubation, alkaline unwinding and electrophoresis proceeded as described in the standard comet assay. Three independent experiments were performed using extracts from different cell cultures.
Results

DNA damage

DNA damage, in the form of SBs and oxidized bases, was measured just after incubation of HeLa and Caco-2 cells with different concentrations of β-cryptoxanthin for 1 h (Figure 1). β-Cryptoxanthin did not cause SBs or base oxidation in HeLa cells (Figure 1A) or Caco-2 cells (Figure 1B). Incubation with β-cryptoxanthin for several hours did not induce any damage (data not shown).

Protection against DNA damage

We tested HeLa and Caco-2 cells for their resistance to DNA oxidation by treating them for 5 min with H2O2 on ice to induce DNA SBs. There were significantly lower levels of DNA breaks in cells preincubated with β-cryptoxanthin compared with control cells (Figure 2A), indicating an enhanced antioxidant defence.

Fig. 1. DNA damage (SBs and FPG-sensitive sites) in HeLa (A) and Caco-2 cells (B) treated for 1 h with different concentrations of β-cryptoxanthin. Standard deviation values based on the results of three independent experiments are shown.

scores of the different groups were compared using the non-parametric Mann–Whitney U-test. \( P \leq 0.05 \) was set as the level of significance.

Cellular repair

The ability of HeLa and Caco-2 cells to rejoin SBs induced by \( \mathrm{H}_2\mathrm{O}_2 \) in the absence and presence of β-cryptoxanthin was followed by measuring the residual damage after different times of incubation (Figure 3). In cells without β-cryptoxanthin, the \( t_{1/2} \) for rejoining of breaks was \( \sim 10 \) min for HeLa and 18 min for Caco-2; in β-cryptoxanthin-treated cells, \( t_{1/2} \) decreased to 4 min for HeLa (both 1 and 4 \( \mu \mathrm{M} \)) and to 10 and 4 min for Caco-2 (1 and 4 \( \mu \mathrm{M} \), respectively). In control cells, the level of SBs was constant during the entire repair incubation time, indicating that preparation and subsequent processing of the cells did not cause significant DNA damage.

We treated cells with Ro plus light to induce 8-oxoGua, and followed BER by measuring residual FPG-sensitive sites over a period of incubation with and without β-cryptoxanthin (Figure 4). Cells treated with β-cryptoxanthin repaired the damage more quickly than untreated cells; in HeLa cells, the \( t_{1/2} \) of \( \sim 135 \) min without β-cryptoxanthin decreased to 80 and 65 min with 1 and 4 \( \mu \mathrm{M} \) β-cryptoxanthin, respectively. In Caco-2 cells, the \( t_{1/2} \) decreased from \( \sim 260 \) to 155 and 125 min with 1 and 4 \( \mu \mathrm{M} \) β-cryptoxanthin, respectively.

In vitro repair

The capacity of HeLa and Caco-2 cell extracts for BER of oxidized bases was assessed with and without β-cryptoxanthin pretreatment using an in vitro assay. HeLa and Caco-2 cells were incubated for 2 h with 0, 1 or 4 \( \mu \mathrm{M} \) β-cryptoxanthin before preparation of a whole-cell extract. Extracts were then incubated with substrate nucleoids containing 8-oxoGua or without lesions, and incision was detected using the comet assay. Mixed extraction plus reaction buffer and FPG were used as negative and positive controls. Figure 5 shows a significant increase in incision by the extracts of cells treated with β-cryptoxanthin in nucleoids containing 8-oxoGua (Figure 5A), even after the low concentration of 1 \( \mu \mathrm{M} \), but no increase in breaks in nucleoids without lesions (Figure 5B).

β-Cryptoxanthin per se did not induce any breaks in nucleoids containing 8-oxoGua or nucleoids without lesions (data not shown).

Western blots

The glycosylase primarily associated with repair of oxidized purines in eukaryotes is OGG1. Removal of the base is followed by cutting at the apurinic site by APE1 (17). The increased BER of 8-oxoGua might be due to an increase in cellular concentration of these proteins. We therefore carried out western blots on whole-cell extract and nuclear extract from HeLa and Caco-2 cells with and without pretreatment with β-cryptoxanthin. Results are shown in Figure 6. APE1 was readily detected in both whole-cell and nuclear extracts. hOGG1 was much less intense and was especially difficult to detect in whole-cell extract (therefore only results with nuclear extract are shown). After normalizing band intensities against the reference anti-GAPDH, only minor differences were seen (the most pronounced being an \( \sim 50\% \) decrease in anti-APE1 intensity in whole-cell extract from Caco-2 cells with β-cryptoxanthin). We conclude that the increase in OGG activity of the cell extract is not related to a detectable increase in enzyme concentration.
Experiments are shown.

In an interesting contradictory study, Bianchini et al. (22) investigated groups of women in Spain and Sweden and found significant positive correlations between 8-oxodGuo in lymphocyte DNA and cancer survivors; there were negative correlations with several plasma carotenoids, including \( \beta \)-cryptoxanthin. It should be remembered that, in association studies of this sort, plasma carotenoids may simply be a marker of consumption of fruits and vegetables, and some other unidentified components may be responsible for the effect on DNA damage.

In an interesting contradictory study, Bianchini et al. (22) investigated groups of women in Spain and Sweden and found significant positive correlations between 8-oxodGuo in lymphocyte DNA and carotenoid concentrations in plasma.

The results of intervention trials, with carotenoid supplements or carotenoid-rich foods and with DNA oxidation as an end point, have been mixed. Pool-Zobel et al. (23) reported a decrease in pyrimidine oxidation during supplementation with carrot juice. Twelve weeks of supplementation with carotenoids, lutein or lycopene increased plasma concentrations of carotenoids but failed to decrease the level of oxidized bases in lymphocyte DNA (18). Thompson et al. (24) gave a high fruit and vegetable diet for 14 days and found raised plasma carotenoid levels and decreased DNA oxidation in lymphocytes. Van den Berg et al. (25) supplemented volunteers with a vegetable burger and fruit drink for 3 weeks; plasma levels of several carotenoids rose, but there was no change in DNA oxidation. Porrini et al. (26) demonstrated an increased resistance of lymphocyte DNA to \( \text{H}_2\text{O}_2 \)-induced breakage after supplementation with tomato puree (rich in lycopene).

In our experiments applying \( \beta \)-cryptoxanthin to cells in culture, there was a clear, dose-dependent antioxidant-protective effect, decreasing the breaks induced by \( \text{H}_2\text{O}_2 \) even at a concentration as low as 1 \( \mu \text{M} \) (in the case of Caco-2 cells). Perhaps more impressively, Ro-induced base oxidation was also markedly decreased, in both cell types at 1 \( \mu \text{M} \). The mean plasma concentrations of \( \beta \)-cryptoxanthin vary from country to country and between men and women (27), the highest levels in that study being found in women in Spain (0.42 \( \mu \text{M} \)).

Thus, our effective concentration is close to the physiological range. Bearing in mind that \( \beta \)-cryptoxanthin is one of several carotenoids that probably act as antioxidants in concert, our results are likely to have biological relevance.

Effects of carotenoids on DNA repair have received less attention than antioxidant actions. We reported an apparent enhancement of SB rejoining in lymphocytes isolated from some but not all subjects 24 h after a single large dose of \( \beta \)-carotene, lutein or lycopene (28,29); but we concluded that this effect might simply be an antioxidant-protective effect against additional damage induced by atmospheric oxygen since the freshly isolated lymphocytes were subject to a sudden increase in ambient oxygen concentration.

This consideration does not apply to the cultured cells used in the current investigation. \( \beta \)-Cryptoxanthin at 1 \( \mu \text{M} \) approximately...
doubled the rate at which HeLa and Caco-2 cells rejoin H2O2-induced SBs and had a proportionately similar effect on the intrinsically much slower repair of oxidized bases. That this truly reflects an increase in repair activity is confirmed by the experiments in which cell extracts were incubated in vitro with substrate DNA containing 8-oxoGua; β-cryptoxanthin preincubation of the cells significantly stimulated incision activity. Control experiments exclude the possibility that β-cryptoxanthin itself causes breaks or that it induces non-specific nuclease activity in the extracts. The increase in activity was not explained by any detectable increase in concentration of hOGG1 or of APE1, the two enzymes involved in removing 8-oxoGua and incising the DNA. This agrees with our previous finding that an increase in OGG activity resulting from kiwifruit consumption was not accompanied by any change in expression of hOGG1 or APE1 (30) and with the report of Paz-Elizur et al. (31) that there is a poor correlation between OGG1 activity and hOGG1 messenger RNA levels. It is possible that β-cryptoxanthin is involved in the post-translational regulation of OGG1 or APE1. OGG1 is regulated by phosphorylation (32) and also by acetylation (33), and APE1, as well as being inactivated by phosphorylation (34), is subject to redox regulation by reduction/oxidation at a cysteine adjacent to the active site (35).

Astley et al. (36) treated Molt-17 human lymphocytes (a permanent line) with β-carotene, β-cryptoxanthin or lutein and found no evidence of antioxidant protection against DNA damage, although apparently carotenoids were required for any SB rejoining to occur over a 2 h incubation after H2O2 treatment. There was no sign of an increase in repair activity in an in vitro assay (with oxidation damage in the substrate). Subsequently, lymphocytes from volunteers following various dietary regimes were tested; after a 3 week intervention with cooked carrots (200 g daily), in vitro repair was enhanced, and after mixed carotene capsules, SB rejoining was more rapid (37).
In summary, in our cell culture model system, we report effects of β-cryptoxanthin on levels of DNA oxidation that are consistent with a significant role as an antioxidant in vivo. In addition, β-cryptoxanthin has a striking stimulatory effect on two kinds of DNA repair—SB rejoining and excision repair of oxidized bases. The repair incision activity of a whole-cell extract is also stimulated by this phytochemical. Thus, the cancer-protective role of carotenoids, as seen in observational epidemiological studies, is probably in part at least the result of a stimulation of DNA repair.

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**References**


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**Fig. 5.** *In vitro* DNA repair: incision by HeLa or Caco-2 cell extracts incubated for 10 min with gel-embedded nucleoid DNA containing 8-oxoGuo (A) or nucleoids without lesions (B). Cells were incubated with β-cryptoxanthin (0, 1 and 4 μM) for 2 h before preparing extracts. Standard deviation values based on the results of three independent experiments are shown. *P* ≤ 0.05 (comparison of extracts with and without β-cryptoxanthin preincubation).

**Fig. 6.** Western blots. Top: nuclear extracts; middle: whole-cell extracts; lanes 1–4, anti-APE1; lanes 5–8, anti-GAPDH (reference). Bottom: nuclear extracts; lanes 1–4, anti-hOGG1; lanes 5–8, anti-GAPDH. Lanes 1 and 5, HeLa cells untreated; lanes 2 and 6, HeLa cells pretreated with β-cryptoxanthin. Lanes 3 and 7, Caco-2 cells untreated; lanes 4 and 8, Caco-2 cells pretreated with β-cryptoxanthin. After normalizing band intensities with reference to GAPDH, ratios of intensities with/without β-cryptoxanthin were: nuclear extract: APE1, HeLa, 0.8; APE1, Caco2, 0.8; hOGG1, HeLa, 1.2; hOGG1, Caco2, 1.0. Whole-cell extract: APE1, HeLa, 1.3; APE1, Caco2, 0.5.

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