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Lycopene and β-carotene Protect against Oxidative Damage in HT29 Cells at Low Concentrations but Rapidly Lose this Capacity at Higher Doses

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Epidemiological studies have clearly demonstrated a link between dietary carotenoids and the reduced incidence of certain diseases, including some cancers. However recent intervention studies (e.g. ATBC, CARET and others) have shown that β-carotene supplementation has little or no beneficial effect and may, in fact, increase the incidence of lung cancers in smokers. This presents a serious dilemma for the scientific community – are carotenoids at high concentrations actually harmful in certain circumstances?

Currently, a significant number of intervention studies are on-going throughout the world involving carotenoids (of both natural and synthetic origin). Our approach has been to study the ability of supplementary carotenoids in protecting cells against oxidatively-induced DNA damage (as measured by the comet assay), and membrane integrity (as measured by ethidium bromide uptake). Both lycopene and β-carotene only afforded protection against DNA damage (induced by xanthine/xanthine oxidase) at relatively low concentrations (1–3 μM). These levels are comparable with those seen in the plasma of individuals who consume a carotenoid-rich diet. However, at higher concentrations (4–10 μM), the ability to protect the cell against such oxidative damage was rapidly lost and, indeed, the presence of carotenoids may actually serve to increase the extent of DNA damage. Similar data were obtained when protection against membrane damage was studied.

This would suggest that supplementation with individual carotenoids to significantly elevate blood and tissue levels is of little benefit and, may, in fact, be deleterious. This *in vitro* data presented maybe significant in the light of recent intervention trials.

Keywords: β-carotene, lycopene, comet, xanthine oxidase

INTRODUCTION

There is good evidence to suggest an inverse correlation between the intake of fruit and vegetables and the incidence of certain cancers in humans.[1–3] Dietary antioxidants present in these foods are thought to decrease free radical attack upon DNA. One such group of antioxidants is the carotenoids. The four major dietary carotenoids found in plasma are β-carotene, lycopene, lutein and β-cryptoxanthin. The biological activities of these carotenoids include the

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scavenging of peroxyl radicals, induction of cell–cell communication and the inhibition of proliferation of transformed cell lines.[4–7] Unlike β-carotene and β-cryptoxanthin, lycopene does not exhibit any provitamin A activity, however lycopene oxidation products have been found in human serum and these are thought to confer some biological activity.[8,9] Lycopene may also protect against the onset of prostate cancer, as demonstrated by both *in vivo* and *in vitro* studies.[10,11]

Following the outcome of recent intervention trials β-carotene has attracted some adverse publicity in its role as a cancer chemopreventative agent. The Alpha Tocopherol, Beta-Carotene cancer prevention study (ATBC)[12] indicated that male Finnish smokers who received β-carotene had an 18% increase in the incidence of lung cancer. This outcome was confirmed in the 1996 US Carotene and Retinol Efficiency Trial (CARET) study,[13] in which a group of smokers receiving the combination of β-carotene and retinol had a 28% increase in the incidence of lung cancer, compared to the control group. In addition, the large U.S. Physicians Health Study[14] indicated that there was no overall effect of supplemental β-carotene on lung or other cancers.

Experimental evidence presented by Truscott[15] and Liebler[16] suggests that the gas phase of smoke can react with β-carotene to form radical adducts, and that such species can react with oxygen to form a carotene peroxyl radical that has a prooxidant activity. Cigarette smoke contains both ROS and RNS species, with hydrogen peroxide being present in the breath of smokers.[17] Hydrogen peroxide is a mild oxidant, and in a recent study it interacted with β-carotene supplemented cells to produce greater DNA damage than in the control cells (J.A. Woods, R.F. Bilton and A.J. Young, unpublished data). However when the supplemented cells were challenged with tert-butyl hydroperoxide then protection against DNA damage was observed in the carotenoid supplemented cells. This demonstrated that β-carotene supplemented cells respond differently to the nature of the oxidative challenge they are exposed to. It also indicated that hydrogen peroxide (a constituent of cigarette smoke) may have a role in the detrimental effects of β-carotene observed in the ABTC and CARET studies.

During inflammatory episodes in the gut, cells may be exposed to free radicals including superoxide and hydrogen peroxide generated by neutrophil activation. In this study we investigated the effect of β-carotene and lycopene on free radical-mediated DNA and membrane damage in HT29 cells. A mixture of superoxide and hydrogen peroxide was produced using a xanthine/xanthine oxidase reaction. Both carotenoids displayed opposing effects in terms of protecting HT29 cells from such free radical-mediated damage: lycopene and β-carotene afforded significant protection at lower concentrations (1–3 μM) but at higher concentrations the ability of these two compounds to protect the cells rapidly declined. The possible implications of supplementation with carotenoids and their role in cancer chemoprevention is discussed.

MATERIALS AND METHODS

Cell Culture

HT29 cells derived from an adherent colon carcinoma cell line were maintained at 37°C in an atmosphere of 5% CO_2, 95% O_2. The culture medium RPMI was supplemented with 10% foetal calf serum. When the cells were required for experimental procedures, they were washed once with PBS prior to treatment with 1 ml of 0.25% w/v trypsin-EDTA (Sigma) to detach them from the tissue culture flask surface. Detached cells were plated onto 35-mm diameter petri dishes at approximately 2×10^5/ml and allowed to adhere to the petri dish surface overnight at 37°C.

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Carotenoid Preparation

Carotenoids were initially dissolved in a small volume of dichloromethane to ensure the complete dispersion of carotenoid crystals. This extract was eluted through a short neutral alumina column (Brockman Grade III), using hexane. The coloured fraction obtained was dried under a stream of oxygen free nitrogen, dissolved in hexane and the concentration was determined by UV/Vis spectroscopy using published extinction coefficients.[18] The carotenoid extracts were then dried under a stream of oxygen-free nitrogen and stored at −80°C.

Free Radical Generation

The medium was removed from the HT29 cells, and replaced with 1 ml of fresh RPMI. The purified carotenoids were dissolved in tetrahydrofuran (THF), and added to the petri dishes at concentrations ranging from 1 to 10 μM, such that the concentration of THF never exceeded 0.5% (v/v). The cells were then incubated at 37°C in an atmosphere of 5% CO_2 and 95% O_2 for a further 2 h. Control plates lacking carotenoid were co-incubated with the test plates.

The cells were then washed to remove any unbound carotenoid, and then challenged with xanthine/xanthine oxidase. Superoxide generation by this enzyme system was determined using a cytochrome c reduction assay.[19] The reaction volume was 1 ml and consisted of 0.8 ml PBS, 160 μM xanthine and the reaction was initiated by the addition of 20 mU xanthine oxidase. In certain experiments 50 U/ml SOD or 22 μg/ml of catalase was added to the reaction mixture. The petri dishes were incubated at 37°C for 10 min. The plates were then washed and the medium replaced with 1 ml of PBS. The cells were then removed from their adherent surface by use of a cell scraper to gently detach the cells from the surface of the dish.

Viability Studies

The viability of the cells was assessed by Trypan blue exclusion. To distinguish healthy cells from apoptotic cells, they were stained with ethidium bromide and acridine orange,[20] and the cells visualised under a fluorescence microscope using a blue filter. The healthy cells stain green and the apoptotic ones orange.

Lactate Dehydrogenase

Following lycopene uptake by the cells and challenge with xanthine/xanthine oxidase, the medium was removed. Lactate dehydrogenase activity was determined in the medium using a kit from Sigma. These results were compared with total LDH obtained from 2×10^5 cells following lysis of the cells at 4°C with 0.5% (v/v) Triton X-100 in PBS supplemented with 50 μM PMSF.

Comet Assay

The comet assay was performed essentially as described by Booth *et al.*[21] Briefly, 85 μl of molten 1% normal agarose in PBS was dropped onto a pre-coated microscope slide, covered with an 18 × 18 mm No. 1 glass coverslip and left on ice to set. Once set the coverslip was removed. The HT29 cells were then mixed with 85 μl of 1% low melting point agar and immediately pipetted onto the layer of agarose on the slide. The coverslip was replaced and allowed to set on ice. The entrapped cells were then lysed in 150 ml of ice cold lysis buffer (2.5 M NaCl, 83 mM EDTA, 10 mM TRIS) and the pH was adjusted to 10 using sodium hydroxide. The lysis buffer is supplemented with 1% (v/v) Triton X-100 and 10% (v/v) DMSO prior to use. Lysis was performed at 4°C for 60–90 min. Following lysis the slides were incubated in electrophoresis buffer (0.3 M NaOH and 1 mM EDTA) for 20 min prior to electrophoresis. Electrophoresis was performed at 20 V/32 mA for 24 min. The slides were first

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washed in 100 mM TRIS, pH 7.5, and were then stained with ethidium bromide. The cells were then analysed using a Nikon fluorescence microscope in conjunction with a Kinetica imaging software package. The parameter used to measure DNA damage in this study was the relative tail moment. This refers to the amount of DNA in the tail and the mean distance migrated by the tail, divided by the negative control value. Negative controls were determined using (a) untreated cells, (b) cells that had been exposed to THF and (c) carotenoid supplemented cells alone or in the presence of xanthine oxidase or xanthine. All the negative controls gave very similar tail moments. The appropriate negative control was employed to calculate the relative tail moment for each experiment. A frequency distribution of the tail moment was determined for each test. The mean relative tail moments were presented using the mean data from three experiments scoring 50 cells per slide. The difference distribution of tail moments was analysed using the Mann–Whitney test.

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Carotenoid Uptake by HT29 Cells

HT29 cells were incubated with the relevant concentration of carotenoid for a 2 h period under the appropriate conditions. The medium was removed and the cells were washed twice in PBS, and following scraping, the cells were placed in a glass vial. The carotenoids were extracted by the addition of 1 ml of methanol, 1 ml of 1 M NaCl followed by the addition of 1.5 ml of diethyl ether and 1.5 ml of hexane. The sample was mixed and the phases were allowed to separate. The upper organic phase was removed and dried under a stream of nitrogen at 30°C. A Rheodyne injection system was used to inject 20 μl of sample onto an ODS2 reversed phase column (Spherisorb 25 × 0.46 cm). The carotenoids were eluted using an isocratic solvent system comprising of acetonitrile : THF : methanol (68 : 22 : 10 v/v/v), with 0.025% (w/v) ammonium acetate at 1 ml/min using a LKB Pharamacia 2165 pump. Carotenoids were detected at 460 nm by a Spectra-Physics Spectra Focus scanning wavelength detector. Integration was performed using Spectra Physics software.

Spectroscopic Analysis of Carotenoids

One ml of 2 μM solution of carotenoid in THF was added to a glass vial, and the solvent was evaporated under a stream of oxygen-free nitrogen. The pigment was then challenged with xanthine/xanthine oxidase. The vial was incubated at 37°C for 10 min in the presence of 1 ml PBS containing 160 μM xanthine and 20 mU of xanthine oxidase. The carotenoid was then extracted by the addition of 1 ml ethanol, followed by the addition of 1.5 ml of diethyl ether and 1.5 ml hexane. Upon mixing the two layers were allowed to separate, the upper organic layer was removed and dried under a stream of oxygen-free nitrogen. The extracted carotenoid was then dissolved in 1 ml of hexane, and the electronic absorbance spectrum obtained between 300 and 800 nm using a Milton Roy MR3000 diode array spectrophotometer. This approach was adopted to avoid any possible interference of THF with hydrogen peroxide mediated carotenoid interactions. In the cellular experiments THF was removed by washing prior to challenging the supplemented cells with xanthine/xanthine oxidase.

RESULTS

Cell Viability

The initial viability of the HT29 cells was 97 ± 0.34% as estimated by Trypan blue exclusion. Following free radical attack this slightly decreased to 95 ± 0.43% (t-test: $p > 0.05$; $n = 6$ non-significant difference). Cell viability was also assessed using acridine orange/ethidium bromide uptake. Cells that fluoresce green are healthy whereas cells that fluoresce orange/red

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are either apoptotic or necrotic. The presence of THF alone did not significantly enhance the uptake of ethidium bromide (*t*-test: $p > 0.05$; $n = 6$) by the control cells. Cells that fluoresced red increased from $20.3 \pm 1.2\%$ to $37.7 \pm 3.1\%$ (*t*-test: $p < 0.05$; $n = 6$) following xanthine/xanthine oxidase treatment. The number of necrotic and apoptotic cells was determined by the Tunel assay, and this indicated that $< 10\%$ of the cells were damaged in this way. The discrepancy between values for Trypan blue exclusion and ethidium bromide uptake may be accounted for due to the difference in sizes of the two molecules. Furthermore the apoptotic cells take up ethidum dye, but fail to take up Trypan blue.

Following supplementation with either lycopene or β-carotene the viability of the HT29 cells was found to be very similar to that of the non-supplemented cells. In the case of lycopene, the percentage of cells exhibiting Trypan blue exclusion was $94 \pm 0.75\%$ and fluorescing red following ethidium bromide/acridine orange uptake was $18.3 \pm 0.72\%$. For β-carotene the percentage of viable cells was $95 \pm 0.25\%$ as assessed by Trypan blue exclusion, and $19 \pm 0.63\%$ demonstrated ethidium bromide uptake.

The integrity of the cell membrane was examined by lactate dehydrogenase release. Less than 10% of the total cellular LDH was released following xanthine/xanthine oxidase treatment. This result together with the Tunel assay would suggest that the cells were permeabilised following free radical attack.

Carotenoid Uptake Studies

The carotenoids were delivered to cells dissolved in THF as we were unable to obtain sufficiently high concentrations of carotenoids in liposomal-micellar preparations to give an adequate concentration range for the dose–response experiments.

The analysis of carotenoids particularly lycopene, was extremely difficult to perform in this study. Reproducible uptake values of between 10% and 15% were obtained at 1 and 5 µM. At concentrations > 5 µM less reliable results were obtained with apparent carotenoid aggregation and crystallisation on the cell monolayers. The solubility of the carotenoids in THF/RPMI may be partially responsible for the inflection in the dose–response observed between 5 and 10 µM (Figures 2 and 3).

Carotenoid Supplemented Cells Challenged with Xanthine/Xanthine Oxidase

The supplemented HT29 cells were challenged with xanthine/xanthine oxidase for 10 min at 37°C. Although the majority of unbound carotenoid was removed by extensive washing the residual carotenoid in the incubation mixture did not affect the activity of the xanthine oxidase. The rate of superoxide production was not diminished and neither was the activity of the enzyme when determined by monitoring uric acid production (G.M. Lowe and R.F. Bilton, unpublished data). Following ROS challenge, the cells were then examined for single strand DNA breaks using the comet assay. The software for the comet analysis determines the tail moment; the frequency of distribution of the tail moments is presented in Figure 1. This shows a non-normal distribution of the tail moment, and indicates varying degrees of DNA damage in the population of cells screened. It can also be seen (Figures 1 and 2) that the cells treated with either lycopene or β-carotene exhibit protection against DNA damage only at the lower doses of carotenoid used. As the carotenoid concentration increases so does the DNA damage leading to a reversal of its protective role observed at lower concentrations (Figure 2). At 10 µM both lycopene and β-carotene failed to demonstrate any degree of protection against DNA damage and the levels of damage were similar to that of the positive control. Studies with antioxidant enzymes (Table I) indicated that hydrogen peroxide and not superoxide was largely responsible for both

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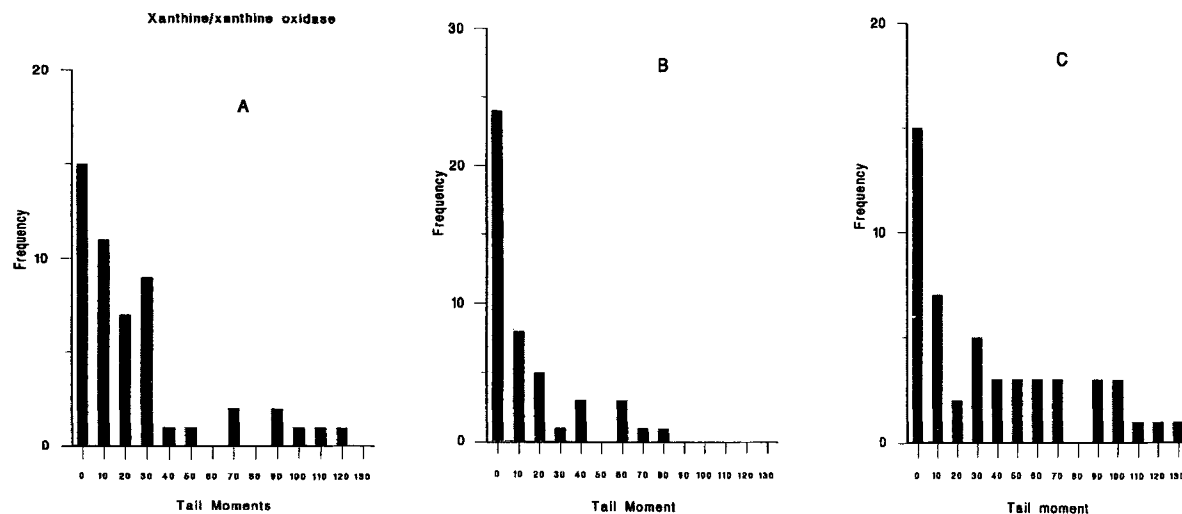

FIGURE 1 HT29 cells were supplemented with 2 and 5 μM β-carotene. The cells were challenged with ROS generated by xanthine/xanthine oxidase. DNA damage was assessed using the alkaline comet assay. During this assay 50 cells were examined for each sample, and the frequency of the tail moments were recorded. (A) Non-supplemented HT29 cells challenged with xanthine/xanthine oxidase. (B) Cells supplemented with 2 μM β-carotene, then challenged with xanthine/xanthine oxidase and (C) HT29 cells supplemented with 5 μM β-carotene, and challenged with xanthine/xanthine oxidase. Analysis using the Mann–Whitney test revealed that there is a difference in the distribution of tail moments between the positive control (A) and the 2 μM β-carotene sample (B); ($p < 0.05$). The test also revealed that the distribution between the control (A) and 5 μM β-carotene (C) is not significant ($p > 0.05$). Data presented were obtained from one experiment. Subsequent data for both β-carotene and lycopene were similar.

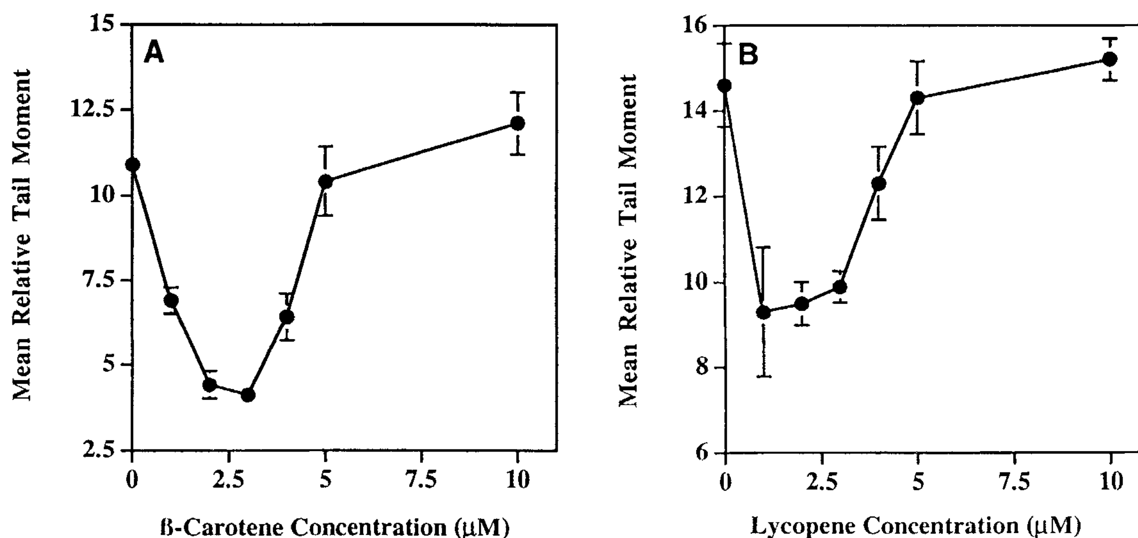

FIGURE 2 HT29 cells were incubated with β-carotene or lycopene. Following washing to remove any unbound carotenoid, the cells were challenged with ROS generated by xanthine/xanthine oxidase. DNA damage was then assessed using the comet assay under alkaline conditions, to detect single and double strand breaks. The mean relative tail moment derived from three separate experiments is presented for each dose of the carotenoid used in this study.

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membrane and DNA damage; i.e. catalase largely protected the cell against DNA and membrane damage while SOD failed to provide any protection (see Table I).

The failure of either lycopene or β-carotene to protect cells from DNA damage when challenged with xanthine/xanthine oxidase was mirrored in membrane integrity studies using acridine/ethidium bromide uptake (Figure 3).

TABLE I DNA and membrane damage by ROS species generated by xanthine/xanthine oxidase

Treatment	RTM	% EtBr
Xanthine\xanthine oxidase (X\XO)	10.5 (0.5)	45.3 (5.5)
X\XO + CAT	1.5 (0.6)*	25.3 (1.9)*
X\XO + SOD	8.2 (0.5)	33.6 (8.7)
X\XO + SOD + CAT	1.5 (0.6)*	29.3 (6.4)*
Xanthine (control)	0.9 (0.4)*	24.7 (1.6)*

HT29 cells were challenged with xanthine/xanthine oxidase for 10 min at 37°C in the presence of either 50 U SOD or 22 μg/ml catalase. The cells were detached by gentle scraping, and DNA damage was assessed using the comet assay, and membrane damage assessed by ethidium bromide uptake. The data suggests that hydrogen peroxide was largely responsible for both DNA and membrane damage. The data shown is the mean of three experiments (± SEM). The values marked as * indicate that the means are significantly different from the positive control ($p < 0.05$).

The number of apoptotic and necrotic cells present was < 10% as indicated by Trypan blue exclusion, the Tunel assay and low LDH release. This would indicate that cells supplemented with higher concentrations (> 4 μM) of carotenoid are permeabilised following free radical attack, and that the extent of such membrane damage is dependent upon the concentration of carotenoid used.

It is interesting to note that there is a high correlation between DNA and membrane damage (Figure 4).

Interaction of Xanthine/Xanthine Oxidase with Lycopene and β-carotene

Xanthine/xanthine oxidase produces both superoxide and hydrogen peroxide.[19] The deleterious effects of these ROS can be seen in solution. Thus, when a dried film of either β-carotene or lycopene was challenged with the xanthine/xanthine oxidase, the overall effect was a rapid bleaching of

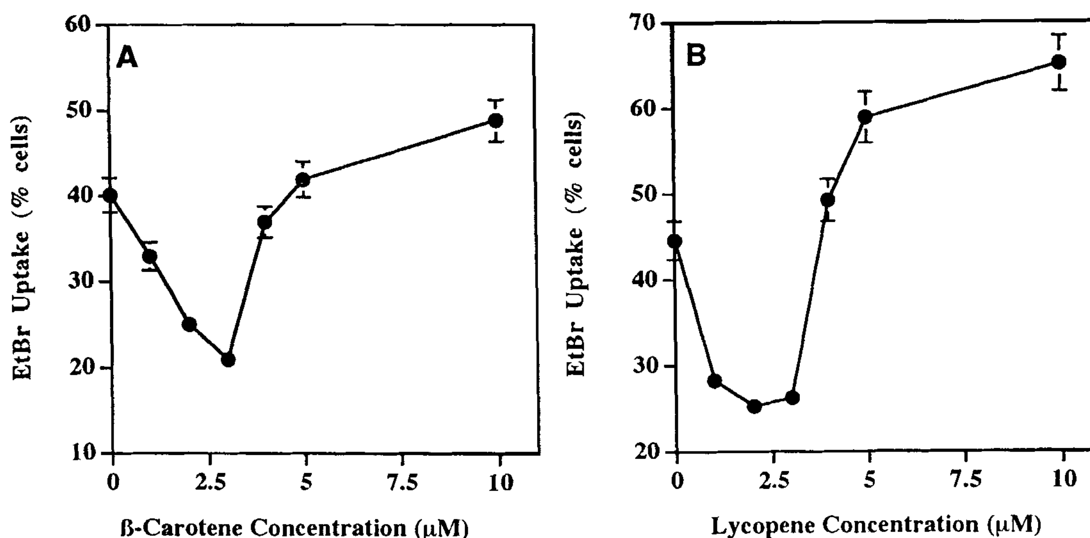

FIGURE 3 HT29 cells were incubated with (A) β-carotene or (B) lycopene. Following washing to remove any unbound carotenoid, the cells were challenged with ROS generated by xanthine/xanthine oxidase. The cells were then stained with acridine orange and ethidium bromide. The cells were then examined under a fluorescent microscope using a blue filter. Two hundred cells were counted and the percentage of red cells was determined. The results presented show results from three separate experiments.

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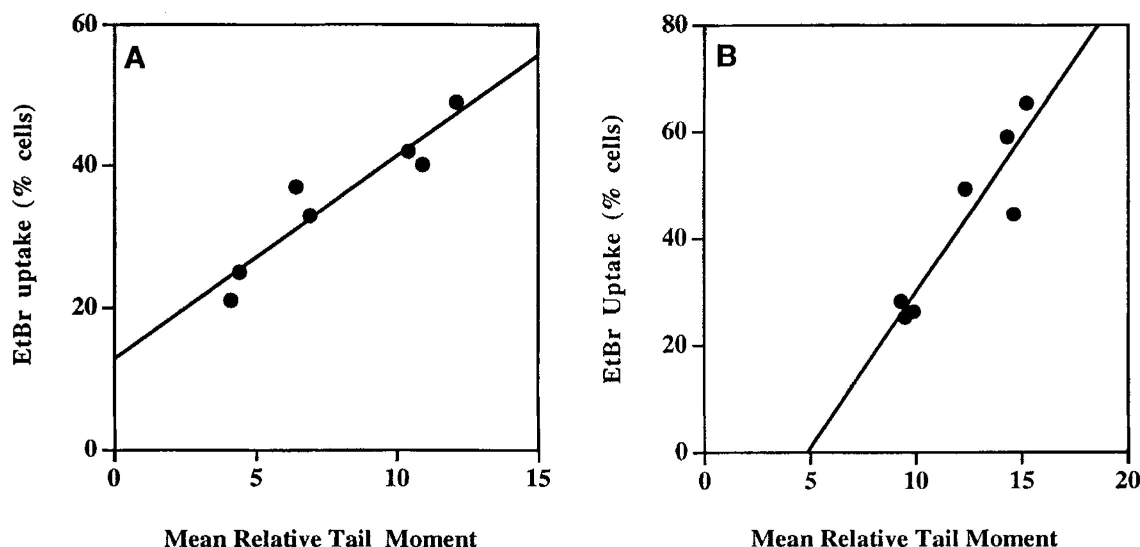

FIGURE 4 Correlation of DNA damage against membrane damage for (A) β-carotene and (B) lycopene. The analysis was performed using cricket graph software. β-carotene: $r^2 = 0.886$, lycopene: $r^2 = 0.848$.

absorbance in the visible region of the spectra (data not shown). This clearly indicates that the products of xanthine/xanthine oxidase reaction can indeed interact directly with both lycopene and β-carotene.

DISCUSSION

ROS Mediated Damage

When HT29 cells were challenged with xanthine/xanthine oxidase significant DNA and membrane damage was observed. Supplementation of HT29 cells with low doses (1–3 µM) of β-carotene or lycopene, resulted in protection from both DNA and membrane damage following ROS challenge. At greater concentrations (4–10 µM) there was a rapid reversal in the protective properties of the carotenoids used in this study (Figures 2 and 3). The reported antioxidant properties of both lycopene and β-carotene are largely associated with singlet oxygen, however some interaction with superoxide has been demonstrated by Truscott.[22] Further to this we demonstrated that the ROS produced by xanthine/xanthine oxidase bleached the colour of dried films of both β-carotene and lycopene. Additional work employing catalase and SOD indicated that hydrogen peroxide was largely responsible for this phenomenon (G.M. Lowe, L.A. Booth, A.J. Young and R.F. Bilton, unpublished data), but there is very limited information available on the possible interactions between a carotenoid and hydrogen peroxide.

β-Carotene can react with alkyl radicals to form a β-carotene radical, and indeed similar lycopene radicals have also been detected.[23] It is thought that this species may interact with α-tocopherol to form a stable β-carotene adduct and the resulting tocopheryl radical being regenerated by ascorbate.[23–25] The partial pressure of oxygen can influence the course of events, in that an increase in the oxygen concentration will tend to favour the formation of a β-carotene peroxyl radical. This species may be responsible for the pro-oxidant effects of β-carotene at the lung surface.[26] It should be borne in mind that incubations prior to cell detachment were all performed in the traditional tissue-culture gas mixture of 95% O_2 and 5% CO_2. This may exacerbate any pro-oxidant activity of the carotenoids. Further studies need to be performed to

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address the problems of oxygen toxicity in tissue culture gas mixtures.

$$\text{L-}\beta\text{C}^{\bullet} + \text{O}_2 \rightarrow \text{L-}\beta\text{COO}^{\bullet} \quad (1)$$

The ability of the carotenoids to act as antioxidants in biological membranes would not only depend upon their physico-chemical properties[27] (i.e. conjugated chain length) but also upon their orientation and the resulting effect upon the plasma membrane organisation. de Ven and colleagues showed that when β-carotene was incorporated into phosphatidylcholine membranes, the orientation of the carotenoid was dependent upon the composition of the lipid bilayers. In the case of dioleyl lecithin bilayers the carotenoids had a parallel orientation and a perpendicular orientation was observed in a soybean lecithin lamellar membranes.[28] The addition of β-carotene to phosphatidylcholine membranes decreased the rigidity of the membrane by increasing the motional freedom of the polar lipid headgroups.[29,30]

When HT29 cells were supplemented with low doses of β-carotene, the carotenoid may act to scavenge radicals in the depth of the membrane and prevent the onset of lipid peroxidation. When greater concentrations were employed the membrane would be expected to become more fluidic and allow greater oxidant damage to occur. Following free radical challenge the cells were not necrotic nor did they release significant amounts of LDH. Ethidium bromide uptake was increased and this indicated that the cells had indeed become permeabilised especially at the higher doses of carotenoids used, this would allow the uptake of small molecules in a non-regulated manner. The consistent trend of protection/deprotection seen in Figures 2 and 3 lead us to speculate that perturbations of membrane organisation may be the crucial factor in controlling membrane permeabilisation and DNA damage when cells were challenged with ROS in this study. These results also indicate that further work is required to establish the relative importance of membrane organisation and radical scavenging capacity of carotenoids at differing concentrations.

Implications

In several recent intervention studies, β-carotene supplementation failed to afford any degree of protection in the subjects, particularly in smokers and asbestos workers. A number of reasons have been suggested by Mayne and others[31–33] e.g. pro-vitamin A activity of β-carotene or the increased dosage of the carotenoid used in the trials. In a 'normal' healthy diet plasma β-carotene levels are 0.3–0.5 µM with lycopene typically higher at 0.5–1 µM.[34,35] In one intervention trial[36] 90 mg of β-carotene was given on a daily basis and such levels can induce plasma concentrations in excess of 5.5 µM.

There is a strong inverse correlation between the intake of fruit and vegetables and the incidence of certain cancers. The human body generally maintains a healthy balance of the different classes of antioxidants. Supplementation of any one component may upset any interaction between the different antioxidants. This may be supported by the recent vitamin C supplementation study.[37] More work needs to be done to understand these interactions. Until then we propose that plasma concentrations of carotenoids and other antioxidants should be maintained through a healthy diet rather than supplementation. In the light of the CARET study the results presented here suggest that increased plasma carotenoid concentrations would afford no protection against ROS. Optimal carotenoid concentrations should therefore be achieved by a healthy diet, but in certain cases there may be a role for carotenoid supplements.

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