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FULL PAPER

Lycopene and beta-carotene ameliorate catechol estrogen-mediated DNA damage

Kaampwe Muzandu, Khlood El. Bohi, Zein Shaban, Mayumi Ishizuka, Akio Kazusaka, Shoichi Fujita*

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Abstract

The consumption of fruits and vegetables is associated with a reduced risk of various ailments, including cancer and cardiovascular diseases. Carotenoids, such as lycopene and beta-carotene, are natural constituents of edible plants and may protect against disease. In this study, the influence of lycopene and beta-carotene on DNA damage caused by catechol-estrogens *in vitro* is examined. One possible mechanism by which catechol estrogens such as 4-hydroxyestradiol (4-OHE_2) and 2-hydroxyestradiol, which cause DNA damage in naked plasmid DNA as well as in cells, contributing to the process of carcinogenesis, is through the generation of reactive oxygen species. It was found that both carotenoids at concentrations ranging from 0.25 to 10 μM significantly inhibit strand breakage induced by 4-OHE_2/copper sulphate by up to～90%in plasmid DNA with beta-carotene being slightly more effective. No prooxidant or cytotoxic effects were observed at the concentrations tested. These carotenoids had a similar, though reduced effect on DNA damage as measured by the comet assay, in Chinese hamster lung fibroblasts. The results obtained show that both lycopene and beta-carotene, most probably and mainly through their potent antioxidant properties, are able to inhibit catechol-estrogen-mediated DNA damage.

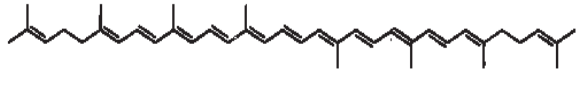
Key words : β-carotene, DNA damage, 4-hydroxyestradiol, lycopene, reactive oxygen species

[1)]Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of VeterinaryInstitutional Address : Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Kita-ku N18W9, Sapporo 060-0818, Japan
*Corresponding Author : Professor Shoichi Fujita
TEL : 81-11-706-6948
Fax : 81-11-706-5105
E-mail : fujita@vetmed.hokudai.ac.jp

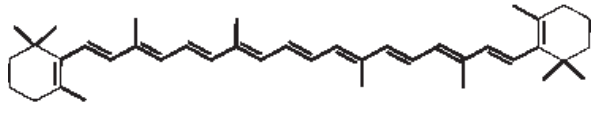
Introduction

Carotenoids are attracting immense interest as possible deterrents to chronic diseases, which include cardiovascular disease and cancers of the prostate, breast and gastrointestinal tract[12, 28, 39]. These compounds are ubiquitous in nature as they are synthesized in plants, microorganisms and algae but must be obtained from the diet in animals and humans. They are responsible for the pigmentation of various fruits and vegetables. For example, the predominant carotenoids found in human tissues, lycopene and β-carotene (fig. 1), impart a red color to tomatoes and an orange color to carrots, respectively. Besides their well recognized function as antioxidants, carotenoids have a wide range of other biological effects such as modulation of the immune response, and induction of gap-junction communications[16, 38].

Most chronic diseases are considered to be caused by oxidative stress[35]. Reactive oxygen and nitrogen species are formed during normal physiological processes in the cell. However, during periods of excessive stress, the natural defense mechanisms of the cells are overwhelmed. Carotenoids in such situations may prove to be invaluable in ameliorating the oxidative stress as they are powerful scavengers of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$) and peroxyl radicals. It has been hypothesized that a probable mechanism of tumor initiation and/or progression by estrogens is through the generation of toxic radicals during the redox cycling of estrogen metabolites[2]. Estrogens may contribute to tumorigenesis in the breast, endometrium, ovary, cervix, brain and prostate[1, 3, 4, 9, 15, 18, 27, 44]. The catechol estrogen, 4-hydroxyestradiol (4-OHE_2), a metabolite of estradiol, is generally believed to be more genotoxic than 2-hydroxyestradiol as it forms depurinating adducts with DNA whereas the latter leads to the formation of stable adducts[2]. Both catechol estrogens undergo redox cycling during which free radicals such as superoxide ($O_2^{\bullet -}$) and the chemically reactive estrogen semiquinone and quinone intermediates are produced. The quinone can bind to DNA to form stable or depurinating adducts with the potential to cause mutations leading to tumorigenesis[2]. Though not very reactive by itself, $O_2^{\bullet -}$ formed during this process is a source of more reactive species such as hydroxyl radicals ($^{\bullet}OH$) and $^1O_2$ (fig. 2).



Lycopene



β-Carotene

Figure 1. Structures of carotenoids

In an earlier study[31], we showed the generation of ROS by 4-OHE_2 in LNCaP/FGC prostate cancer cells sensitized to the glutathione synthesis inhibitor, buthionine sulfoximine. These ROS probably contribute to the DNA damage observed. In the present study, the effect of the carotenoids, lycopene and β-carotene, on DNA damage, an initial event in tumor initiation, caused by 4-OHE_2 is explored. Various concentrations of the carotenoids were used in the in vitro experiments, one of which employed a 4-OHE_2 plus copper system and plasmid pUC18 DNA. In another experiment, DNA damage was as-

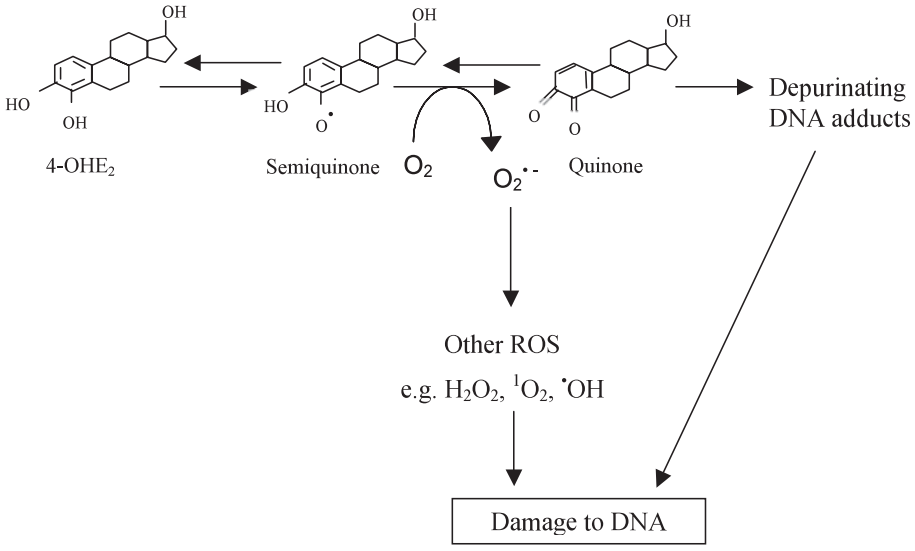


Figure 2. Genotoxicity of 4-hydroxyestradiol

sessed with the comet assay (single cell gel electrophoresis) using a Chinese hamster lung fibroblast (V79) cell line. Physiological concentrations of lycopene and β-carotene found in human plasma range from 0.25 to 1.0 μM[11]. However, following dietary supplementation levels in serum may be as high as 8 and 13 μM[7,34], respectively. In addition, the actual amounts of carotenoids that are taken up by the cells were assessed in order to determine effective intracellular concentrations.

Materials and Methods

Chemicals

4-Hydroxyestradiol and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Chemicals Ltd. (St. Louis, USA). Lycopene, β-carotene and tetrahydrofuran (THF) stabilized with ~0.03% 2,6-di-*t*-butyl-4-methyl-phenol (BHT) were from Wako Pure Chemicals Ltd. (Osaka, Japan). Copper sulphate ($CuSO_4$) was from Kanto Chemical Co., Inc. (Tokyo, Japan).

Stock carotenoid solutions dissolved in THF were kept at -80℃. Working solutions were prepared fresh on the day of an experiment. Exact concentrations were determined spectrophotometrically using established extinction coefficients[36]. In the cell culture experiments, a small amount of stock solution was added to the medium, stirred vigorously and then filtered with a 0.45 μm millipore filter (Billerica, MA, USA). Final concentrations of the carotenoids in the media were measured by spectrophotometry after extraction in *n*-hexane. All procedures were performed under dim light.

Plasmid pUC18 DNA preparation

One Shot TOP10 chemically competent *Escherichia coli* (*E.Coli*) cells (Invitrogen Corp., Carlsbad, USA) were transformed with plasmid pUC18 according to the manufacturer's protocol. Cells were spread on ampicillin (100 μg/ml) agar plates and incubated for 12h at 37℃. Transformed *E. coli* cells were grown in Luria-Bertani medium to a density of approximately 10^9 cells/ml. Plasmid DNA was purified using a Nucleobond® AX100 Kit (Macherey-Nagel, Germany) that uses a modified alkaline/ sodium dodecyl sulfate (SDS) lysis method. Approximately 95% of

the plasmid was in the supercoiled form.

Effect of carotenoids on strand breaks in plasmid DNA

A method described by Li and Trush[20] with some modifications, was used to measure single stranded breaks (ssb) in pUC 18 DNA (2686 bp) by analyzing its agarose gel electrophoretic patterns. Briefly, 0.1 μg of DNA was incubated with different concentrations (0-10 μM) of lycopene or β-carotene and 10 μM 4-OHE_2 plus 20 μM copper sulphate in potassium phosphate buffer (KPB), pH 7.4 at 37℃ for 1 h (final volume 10 μl). Following incubation, 2 μl of electrophoresis loading buffer was added to the reaction mixture which was immediately loaded onto a 1 % agarose gel prepared in Tris Acetate/ethylenediamine tetra acetic acid (EDTA) buffer (40 mM Tris, 20 mM sodium acetate and 2 mM EDTA, pH7.0). Gel electrophoresis was carried out for 2 h at 8.5 V/cm in a horizontal gel electrophoresis apparatus. The gels, pre-stained with 0.5 μg/ml ethidium bromide, were exposed to UV light. Images were taken and scanned, and the DNA strand breaks were measured by the conversion of supercoiled pUC18 double-stranded DNA to open circular and/or linear forms by densitometry. The number of single stranded breaks was calculated as previously described[10].

Effect of carotenoids on DNA strand breaks in cells

Chinese hamster lung fibroblasts (V79 cells) (RIKEN BioResource Center, Tsukuba, Japan) were used in the carotenoid experiments in cells. Cells were cultured in Eagle's Minimum Essential Medium (MEM) containing 10% Fetal Calf Serum (FCS) and antibiotics at 37℃ in 5% CO_2 in air.

Cells were seeded in 35-mm dishes and allowed to attach overnight. They were treated with lycopene, β-carotene or THF for 24 h. The media were replaced, after washing with PBS (phosphate-buffered saline), with media containing either 4-OHE_2 or vehicle (DMSO) and cells were incubated for a further 3 h. DNA damage and cytotoxicity were then determined by the comet assay and by the trypan blue dye exclusion method, respectively. The comet assay was performed according to the method of Singh et al.[37] with some modifications[5]. Briefly, slides were coated with a first layer of 1 % normal agarose. Approximately 20,000 cells were suspended in 50 μl of 0.5% low melting point agarose and layered onto the slides, which were then immediately covered with cover slips. After agarose solidification at 4 ℃ for 5 min, cover slips were removed and slides were immersed for 1 h at 4℃ in fresh lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, and 10 mM Tris, pH 10) containing 1% Triton X-100. The slides were equilibrated in alkaline solution (1 mM Na_2EDTA and 300 mM NaOH, pH>13) for 40 min. Electrophoresis was carried out for 30 min at 1 V/cm. Afterwards, slides were neutralized by washing them three times with 0.4 M Tris buffer (pH 7.5) every 5 min. Slides were stained with ethidium bromide (2 μg/ml). Images were scored using a fluorescent microscope (Olympus BX50 equipped with a 520-550 nm excitation filter). Based on the extent of strand breakage, cells were classified according to their tail length into five categories, ranging from 0 (no visible tail) to 4 (head of the comet still detectable but most of the DNA in the tail). The following formula[24] was used to calculate scores in which N is the number of cells in each category (e.g. N_4 is the number of cells in category 4).

$$\text{Score} = (N_0 + N_1 + 2x\,N_2 + 3x\,N_3 + 4x\,N_4) \times 100 / (N_0 + N_1 + N_2 + N_3 + N_4)$$

Determination of Carotenoid Concentration in Cells

Cells were incubated with various concentrations of carotenoids for 24 h after which the medium was removed and cells washed with PBS. To harvested cells was added 0.5 ml of 10 mM SDS. An aliquot was removed for protein quantification by the method of Lowry et al.[26]. To 400 µl of sample in 10-ml glass test tubes, was added an equal volume of ethanol. The suspensions were mixed for approximately 10 sec on a Vortex mixer. After the addition of 800 µl of n-hexane, the tubes were shaken for 10 min on a shaking device and centrifuged at 2500 g for 10 min. Aliquots (500 µl) of the clear supematant were pipetted into 1.5-ml eppendorf vials and evaporated at room temperature using a Speed-Vac concentrator. The residue was pre-dissolved in 10 µl of dichloromethane, and the solution was diluted with 240 µl of mobile phase and analyzed by HPLC. Chromatographic separation was done as previously described[25].

Statistical Analysis

Differences in means were assessed with the analysis of variance (ANOVA), followed by Fisher's protected least significant difference test. The level of statistical significance was set at $P<0.05$.

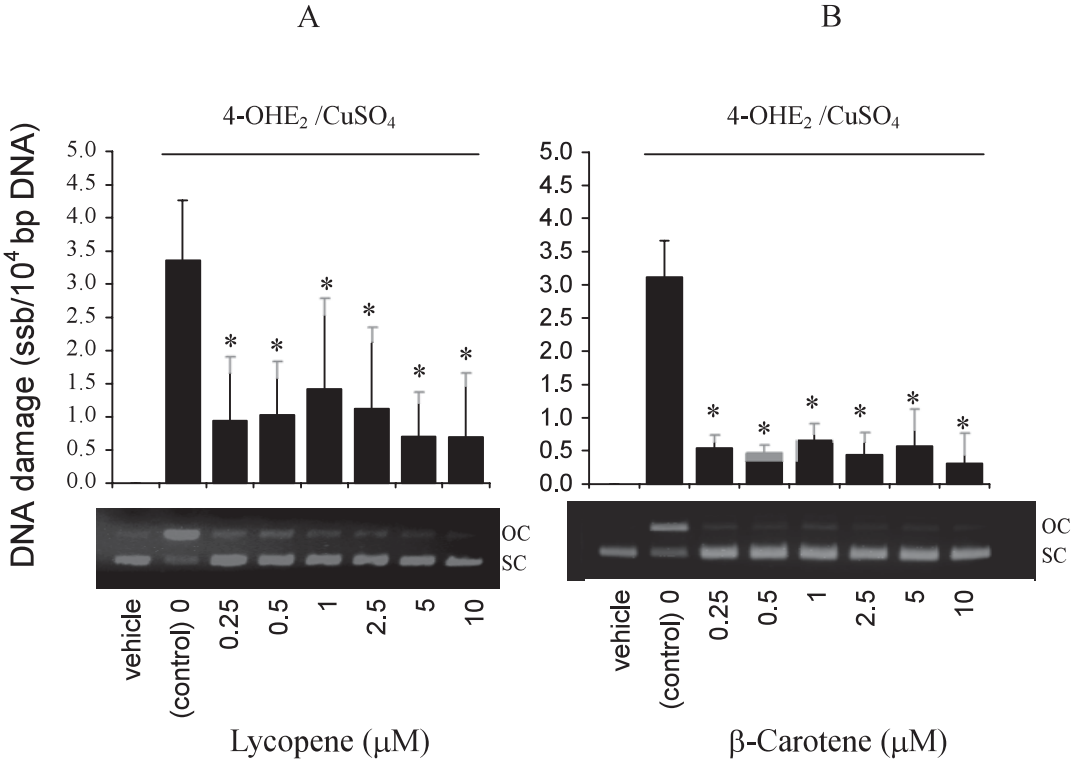


Figure 3. Inhibition of strand breaks by carotenoids in plasmid DNA. pUC18plasmid DNA was incubated for 1 h at 37℃ with the indicated concentrations of lycopene (A) or (β-carotene (B) in the presence of 10 µM 4-OHE_2/20 µM $CuSO_4$ in 0.1 mM KPB, pH 7.4. DNA damage was expressed as single stranded breaks (ssb) per 10^4 base pairs (bp) DNA (upper panels); Representative agarose gel electrophoretic pattern of the DNA after treatment (lower panels) showing conversion of supercoiled (SC) DNA to open circular (OC) DNA. Vehicle, THF/DMSO. Results are presented as means±SD (n = 4). * Significantly different from control, $P<0.05$.

Results

Effect of carotenoids on strand breaks in plasmid DNA

Catechol estrogens cause strand breaks in plasmid DNA in acellular systems comprising metals or enzymes[21-23]. The cause of the strand breaks is believed to be the •OH radical generated during the redox cycling between the catechol estrogen, catechol-semiquinone and catechol quinone species. However, the role of other reactive oxygen species such as $^1O_2$ cannot be ruled out.

Both lycopene and β-carotene strongly inhibited 4-OHE_2/$CuSO_4$-induced strand breakage in plasmid DNA with no significant differences between the different concentrations used (0.25 to 10 μM) (fig. 3A and B). THF alone did not reduce strand breakage, and the catechol estrogen alone did not cause any strand breaks (data not shown). The 4-hydroxyestradiol/$CuSO_4$-induced strand breaks (3.35 ssb/10^4 bp DNA) during lycopene treatment were inhibited by ~64 to 74%. Under our experimental conditions, the damage caused by 4-OHE_2/$CuSO_4$ was not extensive as evidenced by the lack of the linear form of plasmid DNA (fig. 3). In the case of β-carotene treatment, the inhibition was even greater (~82 to 90%). This result is in contrast to a report that relatively low concentrations of carotenoids are more effective in reducing DNA damage while high concentrations actually have prooxidative effects[25].

Effect of carotenoids on strand breaks in cells

A number of studies have shown the genotoxic effects of estrogens or catechol estrogens administered to cells under various conditions[13,19,29,40-42]. In this experiment, V79 cells were treated with 0, 0.312, 0.625, 1.25, 2.5, 5 and 10 μM of carotenoid in the culture medium for 24 h after which they were challenged with 25 μM of the genotoxic chemical, 4-OHE_2. Prior experiments showed that 4-OHE_2 causes a dose-dependent increase in DNA damage after incubation for 3 h (fig. 4A). No loss in cell viability as measured by the trypan blue dye exclusion assay was observed at up to 50 μM of 4-OHE_2 (data not shown). Furthermore, pre-treatment of cells with 0.312-10 μM lycopene or β-carotene followed by 25 μM of the catechol estrogen produced no effects on cell viability (data not shown).

Treatment with increasingly higher concentrations of lycopene resulted in increased protection against DNA damage in cells as shown in fig. 4B. Once again, no prooxidative effects of the carotenoids manifested at these concentrations even though the protection afforded was much less than that observed in the cell-free system (fig. 3). Maximal inhibition (~61%) was obtained with 5 μM-treated cells. As with lycopene, β-carotene provided increased protection against DNA damage as the concentration of carotenoid increased but the effect apparently reached a plateau at 1.25 μM. These results show that carotenoids have the potential to protect cells against DNA damage caused by estrogens and hence also against tumors associated with estrogens.

Carotenoid concentration in cells

Carotenoids tend to be unequally distributed in human tissues with lycopene, for example, being more concentrated in the liver, testes, adrenal glands and prostate than in other tissues[35]. We were interested in finding out to what extent carotenoids were incorporated into V79 cells and if these intracellular concentrations can afford the cells some protection from genotoxic insult by catechol estrogens. The results presented in table 1 show that lycopene was only detectable after the treatment of cells with 2.5 μM or more whereas β-carotene was detectable at a con-

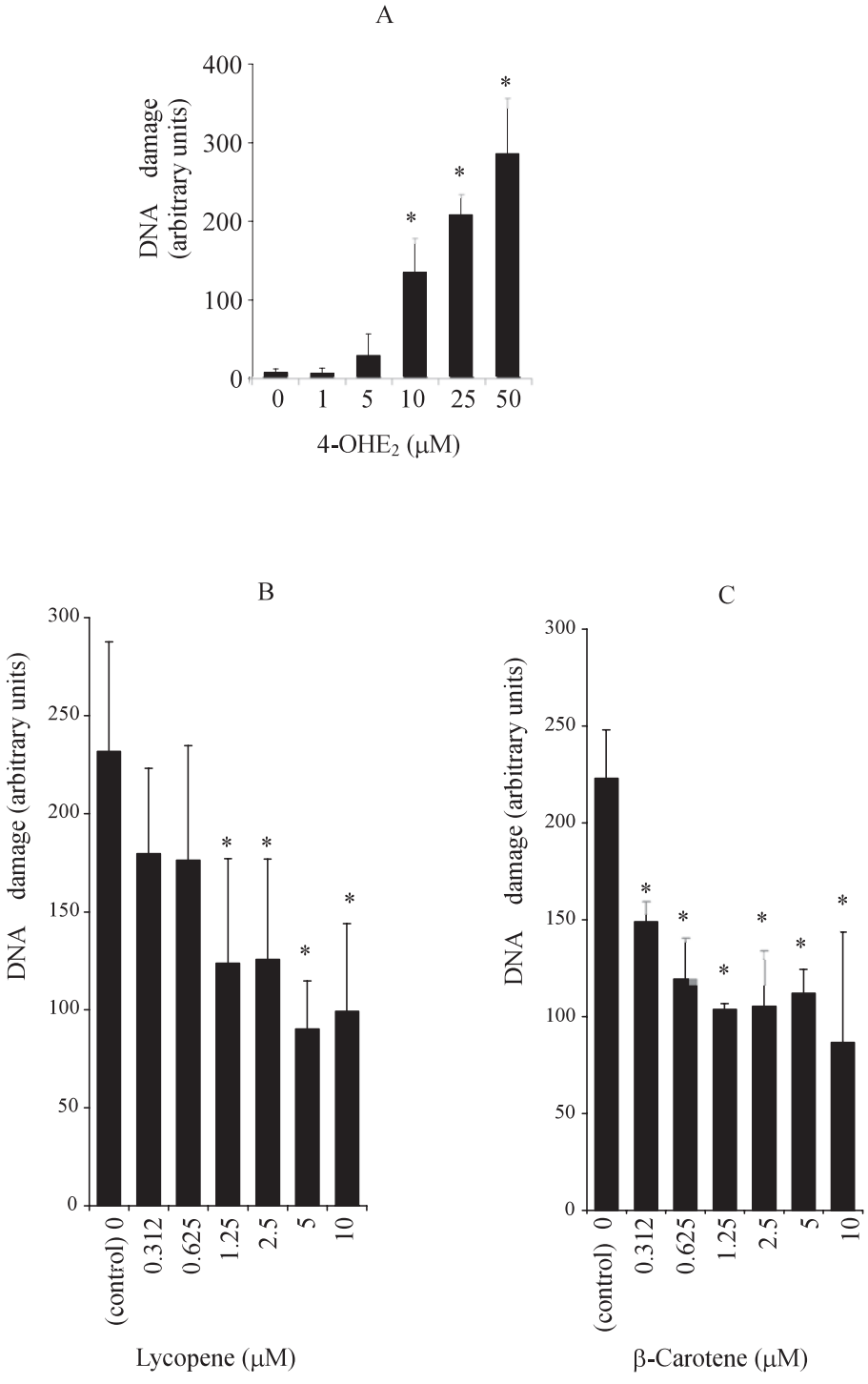


Figure 4. Effect of carotenoids on DNA damage in V79cells. (A) Dose-dependent increase in DNA damage following incubation of cells with 4-OHE_2 alone for 3 h at 37℃ (n= 3). Cells were incubated with media containing indicated concentrations of lycopene (n= 3) (B) or β-carotene (n =4) (C) for 24 h followed by a 3-h incubation with 25μM4-OHE_2 as described in materials and methods. DNA damage was assessed with the comet assay. Results are presented as means ± SD. *Significantly different from 0 μM 4-OHE_2 (A) or control (B and C), $P<0.05$.

centration as low as 0.625 μM. The limits of detection for lycopene and β-carotene were 0.06 and 0.02 nmol/mg protein, respectively.

Discussion

Oxidative stress is regarded as the cause of many cancers and chronic diseases[35,39] and estrogens have been implicated in the etiology of certain cancers. The results obtained in this study indicate that the carotenoids, lycopene and β-carotene, can inhibit the effects of oxidative stress due to estrogens.

As shown in fig. 3, both carotenoids were potent inhibitors of single stranded breakage elicited by the catechol-estrogen, 4-OHE_2, in plasmid DNA. Various concentrations of the carotenoids (0.25-10 μM) were used to determine any concentration-dependent effects on DNA damage. This range reflects concentrations that are found under physiological conditions in human plasma. It has been reported that under certain conditions (e.g. high oxygen tension and/or high carotenoid concentrations), carotenoids act as prooxidants[17,43]. In fact, (β-carotene is associated with an increase in lung cancer in heavy smokers[32]. Furthermore, Lowe et al.[25] found that lycopene and β-carotene were only effective at inhibiting xanthine/xanthine oxidase-induced DNA damage in cells at relatively low concentrations (1-3 μM) with little or no effect at higher concentrations (4-10 μM). In contrast, we found that both low and high concentrations were effective in inhibiting DNA strand breakage (~61-90% inhibition). At just 0.25 μM, the lowest concentration tested, the carotenoids significantly inhibited DNA strand breakage. There were no significant differences between the lower and higher concentrations in the carotenoid treatment groups. In addition, even though β-carotene was generally more effective in inhibiting strand breakage than lycopene, there were no significant differences between the two.

DNA damage was significantly inhibited in V79 cells at 1.25 to 10 μM of lycopene and at all concentrations of β-carotene (fig. 4B). Differences in the pattern of inhibition between the two carotenoids may reflect differences in their cellular concentrations. β-Carotene was detectable over a wider range of concentrations than lycopene (table 1) and this may explain the effectiveness of the former. The differences between lycopene and β-carotene levels in the cells may be due to differences in their physicochemical characteristics, the extent of oxidative degradation in the culture medium, or metabolism. As in the plasmid DNA system, no prooxidative effects of these carotenoids were observed at the concentrations used. There is at the moment no clear explanation for the prooxidative effects at relatively high concentrations observed by some researchers, but the formation of carotenoid aggregates in aqueous media has been suggested[8]. Moreover, prooxidative effects or toxicity, as determined by apoptosis and ROS production, was only observed at much higher and non-physiological concentrations of 50

Table 1

Carotenoid concentration in medium (μM)	Carotenoid concentration in cells (nmol/mg protein)	
	lycopene	β-carotene
0	N.D.	N.D.
0.312	N.D.	N.D.
0.625	N.D.	0.01±0.01
1.25	N.D.	0.51±0.11
2.5	0.57±0.03	0.43±0.05
5.0	0.78±0.06	1.24±0.82
10.0	1.12±0.03	1.48±0.74

Cells were incubated with indicated concentrations of lycopene or β-carotene for 24h (see Materials and Methods). Data are means±SD (n=3). N.D., Not Detected.

and 100 μM in another study[33].

As mentioned earlier, carotenoids are efficient scavengers of singlet oxygen and peroxyl radicals. They have also been reported to scavenge nitrogen dioxide, thiyl, sulphonyl and superoxide radicals[6,30]. Superoxide is generated during the redox cycling of 4-OHE_2 between its semi-quinone and quinone forms and lycopene may prevent DNA damage by scavenging this radical. Furthermore, the antioxidative effects of the carotenoids may also be due to direct scavenging of the semiquinone or quinone radicals. However, it has yet to be demonstrated if this can occur, at least in vitro.

Numerous studies[12,28,39] have shown an inverse association between consumption of fruits and vegetables and cancers of the breast and prostate. It is also worth noting the increasing number of reports linking estrogens to the initiation and/or progression of these cancers[44]. The fact that, lycopene (or fruits and vegetables containing carotenoids and other phytochemicals) is so strongly associated with a reduction in prostate cancer[14,39] indicates that it may be providing protection against estrogen-mediated carcinogenesis. Indeed, in the present study, we found that lycopene and β-carotene prevent DNA damage by catechol-estrogens. In vivo, these and other carotenoids and phytochemicals probably act together to produce enhanced chemopreventive effects against chronic diseases and cancer.

Acknowledgements

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