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Induction of Oxidative DNA Damage in Human Foreskin Fibroblast Hs68 Cells by Oxidized β-Carotene and Lycopene

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Two recent clinical trials suggest that β-carotene may be harmful to smokers. In this study we examined the hypothesis that β-carotene may become toxic when degradation occurs. β-Carotene (BC) and lycopene (LP) with or without prior heat treatment (60 °C for 1 h in open air) were incubated at 20 and 40 μM with calf thymus DNA or human fibroblasts Hs68 cells. The heat treatment resulted in ca. 80% and 35% bleaching of BC and LP, respectively. When Hs68 cells were incubated with the oxidized β-carotene (OBC) or oxidized lycopene (OLP) at 37 °C for 20 h, cell viability was significantly and dose-dependently decreased whereas cell viability was not affected by BC or LP. Cell death, which was already evident at 4 h after incubation with OBC or OLP, was possibly attributable to apoptosis, as shown by the increased histone-associated DNA fragmentation. However, cell lysis, measured as release of lactate dehydrogenase, also occurred at 4 h after incubation with OBC and OLP, although the extent was relatively small and was greater for OLP than for OBC. When calf thymus DNA was incubated with OBC or OLP at 37 °C for 20 h, the 8-hydroxy-2-deoxyguanosine (8-OH-dG) level was significantly and dose-dependently increased by OLP whereas the increase by OBC was only significant at 40 μM. When Hs68 cells were incubated with OBC and OLP for 20 h, both compounds increased the 8-OH-dG level, but the effect was only significant for 40 μM OLP. Comet (single-cell gel electrophoresis) assay of DNA damage in Hs68 cells was determined at 2 h after incubation with OBC or OLP because of its high sensitivity. Both OBC and OLP significantly and dose-dependently increased DNA breakage while BC and LP had no effect. Inclusion of BHT during incubation of cells with 40 μM OBC or OLP partially inhibited (ca. 40%, $p < .05$) the extent of comet formation. Intriguingly, OBC and OLP neither induce lipid peroxidation in Hs68 cells (measured as thiobarbituric acid-reactive substances released into the medium) nor increased the intracellular level of reactive oxygen species. Although it is presently unclear about what degradation products are formed, this study has demonstrated that, when oxidized, BC and LP lead to oxidative damage to both purified DNA and cellular DNA. The results suggest that such damage may contribute to the adverse effects of β-carotene reported in recent clinical studies and caution that it is important to prevent oxidation of BC and LP for human uses such as in supplemental studies.

Keywords: β-carotene, lycopene, autoxidation, DNA damage, 8-OH-dG, comet assay, lipid peroxidation

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Abbreviations: BC, β-carotene; LP, lycopene; OBC, oxidized β-carotene; OLP, oxidized lycopene; 8-OH-dG, 8-OH-2′-deoxyguanosine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; TBARS, thiobarbituric acid-reactive substances; THF, tetrahydrofuran; DMEM, Dulbecco's Modified Eagle Medium; NEAA, non-essential amino acids; ROS, reactive oxygen species; DCFH-DA, 2′,7′-Dichlorofluorescein diacetate; BHT, butylated hydroxytoluene; LDH, lactate dehydrogenase

INTRODUCTION

β-Carotene (BC) and lycopene (LP) are carotenoids that consist of more than 600 natural pigments present in fruits and vegetables.[1] Epidemiological studies have shown inverse relationship between the intake of fruits and vegetables and the risk of several types of cancer,[2] and such effects of fruits and vegetables have been attributed to β-carotene.[3] Both *in vitro* and animal studies have produced supportive results to this theory. Most notably, BC is shown to be an effective antioxidant by scavenging certain ROS, especially peroxyl radical and singlet oxygen, and this antioxidant activity appears to be greatest at low oxygen tension.[4,5] However, two recent clinical trials of supplemental BC, i.e., the ATBC study[6] and the CARET study[7] on the incidence and mortality of lung cancer and cardiovascular disease have produced surprising results, i.e., BC supplementation is either non-protective[8] or even detrimental in smokers.[6,7] Various interpretations of the ATBC[6] and CARET[7] studies have been presented.[5,9–16] Examples of the interpretations are: (1) BC may be toxic at high doses; (2) BC may react with cigarette smoke to generate toxic products; and (3) BC may have been contaminated with toxic degradation products.[5] Additionally, BC has recently been shown to have co-carcinogenic effect by significantly increasing the activities of several carcinogen-metabolizing enzymes (CYP1-A1/2, CYP3A, CYP2B1, and CYP2A) in the lungs of rats supplemented with high doses of BC.[17]

With regard to the toxicity of degradation products of BC, it has recently been suggested that some of these products rather than BC itself may be prooxidative or procarcinogenic.[10] For example, in an experiment involving incubation of calf thymus DNA with rat liver microsomes treated with cytochrome P450-inducing compounds, it was shown that some of the degradation products of BC enhance the binding of benzo[a]pyrene to DNA, although BC itself inhibits the binding.[18] Using human fibroblast Hs68 cells we recently showed that BC and LP, a acyclic non-provitamin A carotenoid with 11 linearly arranged conjugated double bounds, can be either antioxidant or pro-oxidant depending on the type of oxidants used.[19] We also observed that cells incubated with BC or LP alone (i.e., without subsequent treatment with an oxidant) had slightly but significantly increased DNA breakage determined by the single-cell electrophoresis (comet) assay. The results suggest that some degradation products of BC and LP, which may have been formed during incubation, cause damage to DNA. Therefore, in this study we examined the possibility that oxidized BC (OBC) and oxidized LP (OLP) may have pro-oxidant activity in Hs68 cells. To do this, we exposed BC and LP to heat (60 °C) in open air for 1 h before incubation with calf thymus DNA or with Hs 68 cells. DNA damage was determined by comet assay in Hs68 cells and as formation of 8-OH-2′-deoxyguanosine (8-OH-dG) in both calf thymus DNA and Hs68 cells.

MATERIALS AND METHODS

Reagents

All chemicals used were of reagent or higher grade. BC was from Sigma Chemical Co. (St. Louis, MO). LP and tetrahydrofuran (THF) were from Wako Co. (Japan). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin, streptomycin, sodium pyruvate, and non-essential amino acids

(NEAA) were from GIBCO/BRL (Maryland, USA).

Cell Culture

Hs68 cells were cultured in DMEM medium containing 10% (v/v) FBS, 0.37% (w/v) $NaHCO_3$, penicillin (100 unit/ml), streptomycin (100 unit/ml), 0.1 mM NEAA, and 1 mM sodium pyruvate at 37 °C in a humidified incubator under 5% CO_2 and 95% air. The cells were harvested at ca. 90% confluence (ca. 10^6 cells/10-cm dish).

Oxidative Degradation of Carotenoids and Oxidative Treatment of Cells

The purity of commercial BC, as calibrated using an extinction ($E^{1\%}_{450}$) of 2,500 (Sigma Catalog), was ca. 93%, which compared well with the 95% purity claimed by Sigma. For oxidative degradation, BC and LP solutions (10 mM in THF) were incubated at 60 °C in open air for 1 h, based on the spontaneous degradation procedure (60 °C under air or oxygen for 2 h) of BC reported by Handelman *et al.*[20] We shortened the incubation time to 1 h because of the relatively rapid degradation of BC (Figure 1). Degradation of BC and LP was determined as the loss of absorbance at 450 nm and 470 nm, respectively.[21] An aliquot of the carotenoid with or without oxidation was added to confluent Hs68 cells, which were then incubated in DMEM medium containing 0.37% (w/v) $NaHCO_3$, penicillin (100 unit/ml) and streptomycin (100 unit/ml) at 37 °C. THF was added at 0.4% final concentration to serve as solvent control. All incubations were carried out in the dark. After incubation cells were washed twice in phosphate buffered saline (PBS) and the pellet was used for assays of DNA damage. Cell viability was assayed after incubation of cells with OBC or OLP at 37 °C for 20 h using a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay.[22]

Measurement of DNA Damage

8-OH-dG was measured in both calf thymus DNA and Hs68 cells incubated with OBC or OLP. DNA was isolated from Hs68 cells devoid of medium by centrifugation (10,000 × g, 10 min) using phenol/chloroform/isoamyl alcohol.[23] An equal amount of DNA (200 μg) was then digested with nuclease P_1 and alkaline phosphatase, and the 8-OH-dG levels were analyzed by HPLC with an electrochemical detector (Bioanalytical Systems, model LC-4C) as described previously.[24,25] Oxidative damage was expressed as the molar ratio of 8-OH-dG to 10^5 molecules of deoxyguanosine (dG), which was calculated from the absorbance at 260 nm. Comet assay was adapted from the method of Singh *et al.*[26] Cells were suspended in low-melting point agarose in PBS at 37 °C and placed onto a frosted glass microscope slide precoated with a layer of 1% normal-melting-point agarose. After application of a third layer of 1% normal-melting-point agarose, the slides were immersed in cold-lysing solution for 1 h at 4 °C. The slides were then placed in an electrophoresis tank, allowing the DNA to unwind for 15 min in the alkaline solution. Electrophoresis was performed using the method of Collins *et al.*[27] One hundred comets on each slide were scored visually as one of five classes according to tail intensity and given a value of 0, 1, 2, 3, or 4. The total for 100 comets ranged from 0 to 400.

Measurement of Histone-associated DNA Fragmentation

Apoptosis was determined by histone-associated DNA fragmentation test using the cell death detection ELISA kit (Boehringer, Mannheim, Germany).[28] Hs68 cells (ca. 1×10^4) were plated onto a 96-well microplate. After incubation with oxidized or non-oxidized BC or LP for 4 h, cells were centrifuged and lysed in lysis buffer. Following centrifuging at 1,000 × g for 10 min, a portion (20 μl/well) of the supernatant

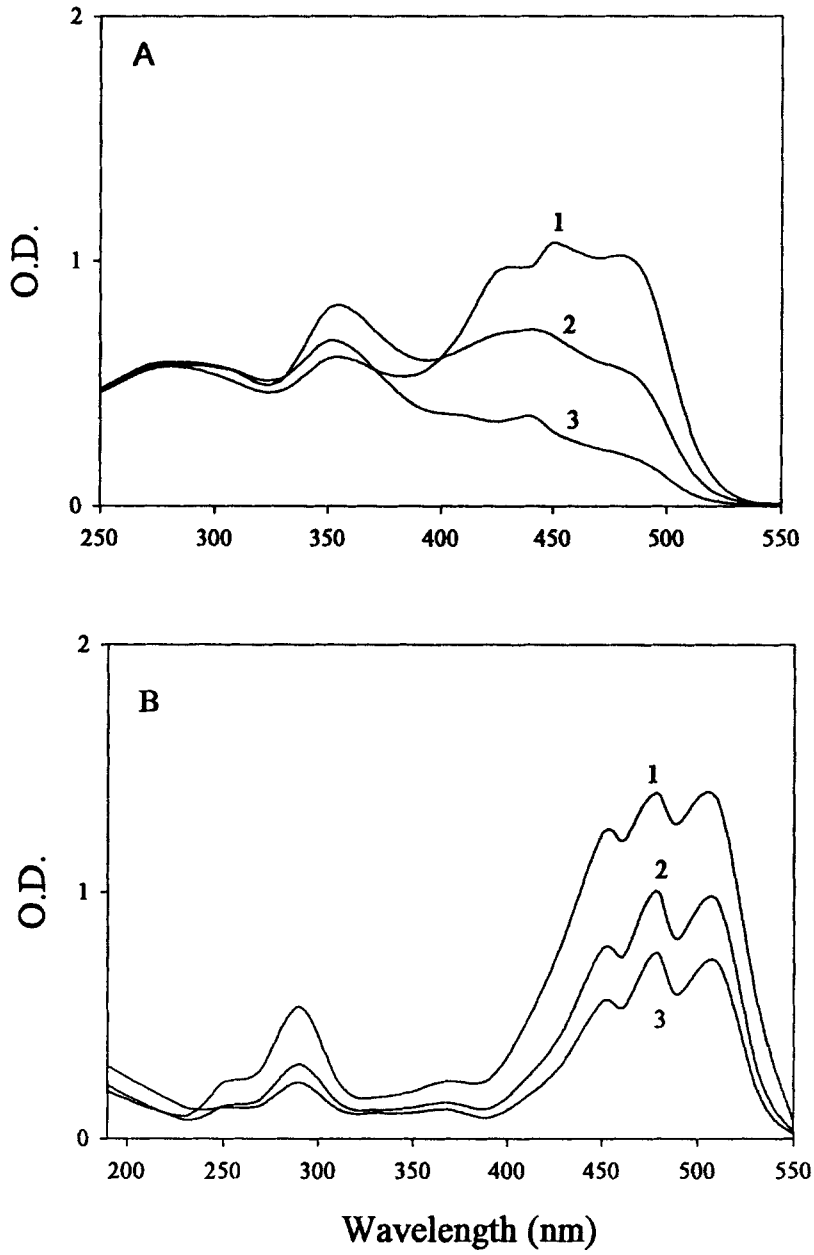

FIGURE 1 Bleaching of β-carotene (A) and lycopene (B) in THF. Numbers 1, 2, and 3 denote absorption spectra recorded after heating in open air at 60 °C for 0, 30, and 60 min, respectively.

was transferred into microplates coated with streptavidin on the bottom. After incubation with immunoreagent containing anti-histone-biotin and anti-DNA-POD for 2 h at room temperature, the substrate solution was added, and the absorbance at 405 nm was measured.

Other Assays

Lipid peroxidation was measured fluorimetrically as thiobarbituric acid-reactive substances (TBARS) released into the medium[29] from Hs68 cells, as described previously.[19] Butylated

hydroxytoluene (BHT at 0.25% final concentration) was included to prevent sporadic lipid peroxidation during heating at 100 °C for 10 min. Generation of intracellular reactive oxygen species (ROS) was assayed using 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), a stable non-fluorescent molecule that readily crosses cell membranes and can be oxidized to highly fluorescent DCF in the presence of intracellular ROS.[30,31] Briefly, 5 μM DCFH-DA was added to cells (ca. 3×10^4) treated with OBC or OLP and incubated at 37 °C in the dark for 1 h. The fluorescence intensity was measured at 485 nm excitation and 530 nm emission.[30,31] Release of lactate dehydrogenase (LDH) was measured as an index of cytotoxicity, and the enzyme activity was measured spectrophotometrically using pyruvate as substrate.[32] Total LDH was measured in cell lysates obtained by treatment with 0.5% Trition X-100, and the percentage of release was determined by dividing the LDH activity in the medium by total LDH activity.

Data Analysis

Values are expressed as means ± SD and analyzed using one-way ANOVA followed by Duncan's multiple range test for comparisons of group means.

RESULTS

Effects of OBC and OLP on Cell Viability

Figure 1 shows the changes in absorption spectra of BC and LP at 30 and 60 min following heat (60 °C) treatment. Heating at 60 °C for 1 h resulted in ca. 80% and 35% loss (i.e., bleaching) of BC and LP, respectively, as determined by the decrease in absorbance at 450 nm (for BC) and 470 nm (for LP). When BC was oxidized at 37 °C, it required ca. 12 h to reach 80% bleaching. The chromatograms from reverse-phase HPLC-photodiode array detector obtained following oxidation of BC at 60 °C for 1 h and at 37 °C for 12 h were very similar, suggesting the formation of similar degradation products between the two oxidation conditions (data not shown).

When Hs68 cells were incubated with OBC and OLP at 37 °C for 20 h, cell viability decreased with increasing concentrations of OBC and OLP (Figure 2). OBC and OLP were equally effective in decreasing cell viability while neither BC nor LP significantly changed the cell viability. The loss of viability induced by OBC and OLP is unlikely due to retardation of cell growth because the cells we used had reached confluence (ca. 90%) and little or no proliferation could occur. We therefore determined the effects of OBC and OLP on cell death, and we found that cell death appeared to be due to apoptosis, as shown by the increased histone-associated DNA fragmentation, and was already evident at 4 h after incubation with OBC or OLP (Figure 3). It should be mentioned that we only found very weak formation of DNA ladder (data not shown). One possible reason for this is that Hs68 cells are quite large and have long doubling time (4–5 days), and consequently, relatively small numbers of cells (and thus small amounts of DNA) are available. In addition to apoptosis, cell lysis measured as release of LDH also occurred at 4 h after incubation with OBC and OLP. However, the percentage release of LDH was relatively minor (3.5 ± 0.4, 5.0 ± 2.2 and 9.0 ± 4.1% for the control, 40 μM OBC and 40 μM OLP, respectively). OBC and OLP led to shrinkage of Hs68 cells, which lost attachment ability and floated in the medium (results not shown).

Effects of OBC and OLP on DNA Damage and Lipid Peroxidation

When calf thymus DNA was incubated with OBC or OLP at 37 °C for 20 h, the 8-OH-dG level was significantly and dose-dependently increased by OLP whereas the increase by OBC was only significant at 40 μM (Figure 4A). In

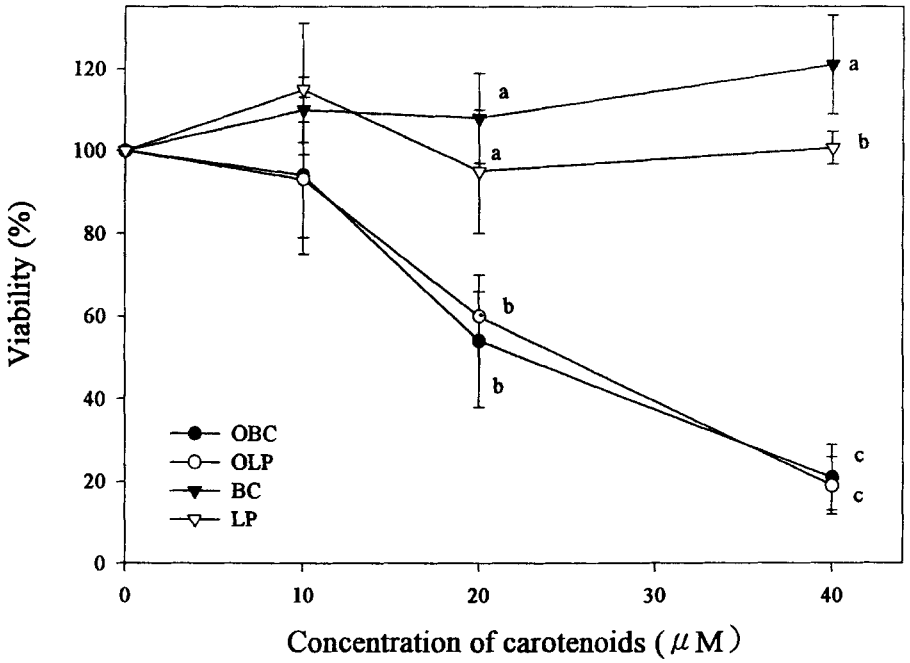

FIGURE 2 The viability of Hs68 cells incubated with β-carotene (BC), lycopene (LP), oxidized β-carotene (OBC), or oxidized lycopene (OLP) at 37 °C for 20 h in the dark. Values (means ± SD of three experiments) at the same concentrations with the different letter are significantly different ($p < .05$).

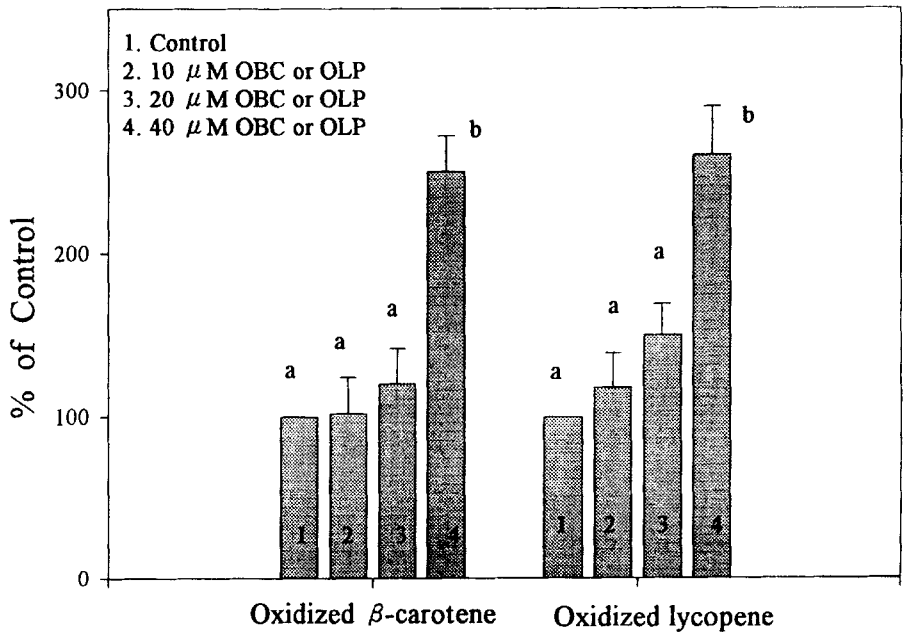

FIGURE 3 Apoptosis of Hs68 cells induced by oxidized β-carotene (OBC) or oxidized lycopene (OLP). Incubation was carried out at 37 °C for 4 h in the dark, and apoptosis was determined as histone-associated DNA fragmentation. Values (means ± SD of three experiments), expressed as relative absorbance of the control (i.e., solvent control, 100%), not sharing a common letter in the same group are significantly different ($p < .05$).

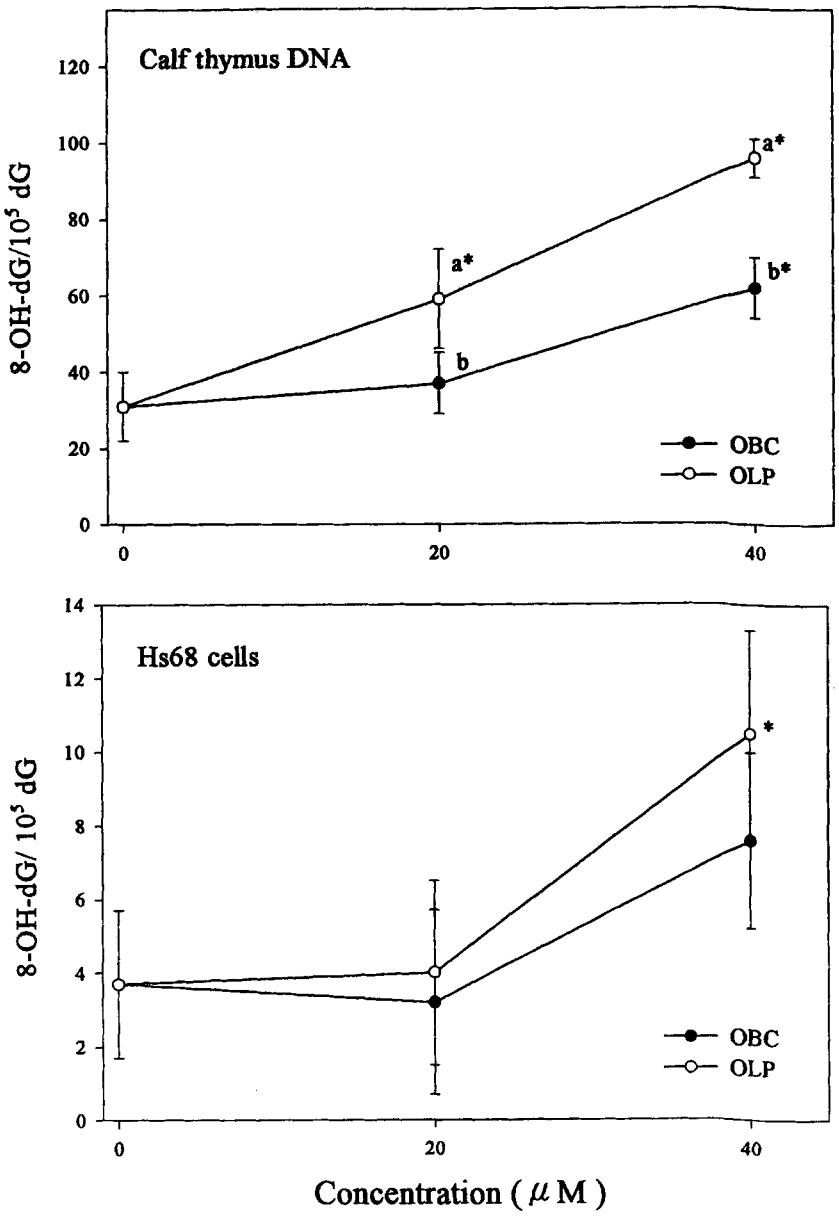

FIGURE 4 DNA damage measured as 8-OH-dG in calf thymus DNA and Hs68 cells incubated with oxidized β-carotene (OBC) or oxidized lycopene (OLP) at 37 °C for 20 h in the dark. Values (means ± SD of three experiments) at the same concentration not sharing a common letter are significantly different ($p < .05$). * Significantly different from the zero-time solvent (tetrahydrofuran) control.

Hs68 cells incubated with OBC or OLP for 20 h, the levels of 8-OH-dG were only increased at 40 μM OBC or 40 μM OLP, and only the increase by OLP was significantly different from the control (Figure 4B).

Comet assay of DNA damage in Hs68 cells, expressed as arbitrary units, was determined at 2 h after incubation with OBC or OLP because of the sensitivity of the assay. Both OBC and OLP significantly and dose-dependently increased

comet formation, and the effect of OLP was greater than that of OBC (Figure 5); at 40 μM, the comet was between classes 3 and 4 for OLP and between classes 2 and 3 for OBC. Inclusion of BHT (0.2 mM final concentration) during incubation of cells with 40 μM OBC or OLP partially inhibited (ca. 40%, $p < .05$) the extent of comet formation; ethanol, in which BHT was dissolved, had no effects at the final concentration used (0.1%). As reported previously,[19] we also found in this study that non-oxidized BC and LP slightly increased comet formation (between classes 1 and 2); the extent of such damage was small for BC but was evident for LP, although the effect of neither compounds was significant.

OBC and OLP did not significantly affect lipid peroxidation determined as TBARS in Hs68 cells (data not shown). It is worth noting that both OBC and OLP (as well as BC and LP) interfere with the TBA test by forming TBARS during the assay and that appropriate controls must be included and subtracted. OBC and OLP also did not significantly affect the levels of intracellular ROS at 4 and 20 h after incubation of the cells with either oxidant (data not shown).

DISCUSSION

To examine the possibility that oxidized carotenoids can damage DNA, we have employed human fibroblast Hs68 cells to study DNA damage induced by BC and LP pretreated by heat at 60 °C under air for 1 h. Our results demonstrate that OBC and OLP induced DNA damage measured as formation of 8-OH-dG and comet (DNA strand breaks in single cells). OBC and OLP also induced cell death that involved possibly apoptosis and slight cell lysis.

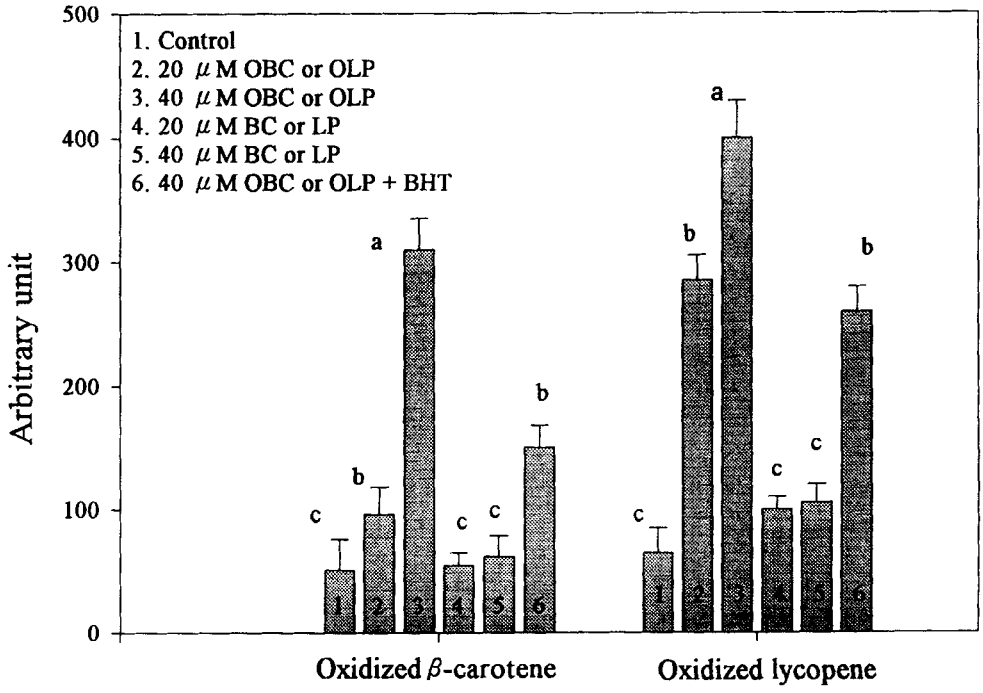

FIGURE 5 DNA damage measured as comet formation in Hs68 cells incubated with β-carotene (BC), lycopene (LP), oxidized β-carotene (OBC), or oxidized lycopene (OLP) at 37 °C for 2 h in the dark. Control: solvent control (tetrahydrofuran, 0.4%); BHT: butylated hydroxytoluene (0.2 mM). Values (means ± SD of three experiments) in the same group not sharing a common letter are significantly different ($p < .05$).

OBC and OLP may cause DNA damage by direct damage from BC radical cation or by indirect damage from their degradation products or from the ROS they produced. Our finding that BHT partially inhibited DNA damage (measured as comet formation) of Hs68 cells induced by OBC and OLP suggests that all these factors are likely to be involved. However, the role of ROS may be minimal since neither OBC nor OLP increased intracellular ROS level. Another possibility is that the BC (and perhaps, LP) radical formed from the reaction with lipid peroxyl radical can damage important cellular macromolecules.[33–36] The BC radical thus formed can be reduced by vitamin E,[5,35,36] and vitamin C, in turn, can reduce vitamin E radical to prevent the damaging effects of BC in smokers.[33] It is well established that cigarette smokers have lower serum levels of vitamin C than do non-smokers and hence, smokers may not have enough vitamin C to prevent such damage. Intriguingly, OBC and OLP apparently did not affect lipid peroxidation determined as TBARS in Hs68 cells (the interference of the assay by OBC and OLP is stated in the Results section). Using a simple linoleic acid system to measure lipid peroxides by an iodometric assay,[37] we also found that OBC and OLP did not affect lipid peroxidation (data not shown). In accord with our findings, Baker *et al.*[38] showed that BC incorporated into a liposomal system followed by exposure to gas-phase smoke mildly inhibits, rather than promotes, lipid peroxidation measured as formation of hydroxy fatty acid methyl esters. In their study, several degradation products of BC were identified, including carotenals, β-carotene epoxides, and 4-nitro-β-carotene.

It thus appears that the oxidatively degraded products of BC and LP are important in causing oxidative damage to DNA. Although it is presently unclear what degradation products are responsible for DNA damage, the results of Handelman *et al.*[20] may provide some clues to OBC (and perhaps, OLP) because we used a relatively similar heat treatment of BC to theirs. By exposing BC to oxygen at 60 °C for 2 h, they identified several carbonyls and epoxides (e.g., β-apo-13-carotene, β-apo-14′-carotene, β-apo-12′-carotene, β-carotene-5, 6-epoxide and β-carotene-15,15′-epoxide). Marty and Berset[39] also identified similar degradation products of BC by prolonged heating at 180 °C. Using reverse-phase HPLC-photodiode array detector, we observed an increase in several degradation products of OBC and OLP that had increased polarity with absorbance between 300 and 350 nm (data not shown), suggesting the presence of β-apo-carotenals and β-carotene-epoxides. Further studies are required to identify the products of OBC and OLP that are responsible for DNA damage.

Our results from comet assay may help explain the seemingly conflicting results of a recent study reported by Lowe *et al.*[40] Using the comet assay to study DNA damage in HT29 cells induced by xanthine/xanthine oxidase, they reported that both BC and LP at relatively low levels (1–3 μM) protect against DNA damage but rapidly lose this capacity, and in fact, may have enhanced such damage at higher concentrations (4–10 μM). Based on our results, it is possible that enough degradation products may have been present at higher levels (4–10 μM) of the two carotenoids.

Our results may have some relevance *in vivo* when comparing the levels of BC and LP incorporated into Hs68 cells with the endogenous levels in human LDL. We reported recently that ca. 1–2% of the BC and LP was incorporated into Hs68 cells in 1 h.[19] For 10^6 cells incubated with 20 μM BC and LP, this is equivalent to 3.5 nmol BC (or 5.8 nmol/mg protein) and 3.8 nmol lycopene (or 6.3 nmol/mg protein). These levels are somewhat higher than the endogenous levels of BC and LP in isolated human LDL (0.22–0.29 nmol BC and 0.37–2.0 nmol LP/mg protein in three subjects).[41] When taking supplements that contain BC, plasma BC levels can be increased greatly. An example is the CARET study,[7] which reported a 12-fold increase in

plasma BC level in subjects supplemented with 30 mg BC/d (in combination with 25,000 IU retinyl palmitate) for five years. It is possible that sufficient amounts of degradation products of BC can exist in the serum and organs of subjects taking BC or LP for a prolonged period of time. Indeed, *in vitro* studies using homogenates or extracts of various tissues from animals and humans have shown that incubation with increasing concentrations of BC lead to increased concentrations of breakdown products that include apo-carotenals.[42,43]

In summary, the present study in calf thymus DNA and Hs68 cells demonstrates that both BC and LP damage DNA when oxidized and that OLP is more damaging than OBC in essentially every assay used. It is unclear what oxidation products are responsible for such damage, but our results suggest that such damage may contribute to the adverse effects of BC reported in recent clinical studies. In particular, our results caution that it is important to prevent oxidation of BC and LP for human uses such as in supplemental studies.

Acknowledgments

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References

[1] S.K. Clinton (1998) Lycopene: chemistry, biology, and implications for human health and disease. *Nutrition Reviews*, **56**, 35–51.

[2] P. Astorg (1997) Food carotenoids and cancer prevention: an overview of current research. *Trends in Food Science and Technology*, **8**, 406–413.

[3] R. Peto, R. Doll, J.D. Buckley and M.B. Sporn (1981) Can dietary beta-carotene materially reduce human cancer rates? *Nature*, **290**, 201–208.

[4] W.G. Burton and K.U. Ingold (1984) β-Carotene: an unusual type of lipid antioxidant. *Science*, **224**, 569–573.

[5] B. Halliwell and J.M.C. Gutteridge (1999) *Free Radicals in Biology and Medicine*, Third edn. Oxford University Press, New York, pp. 223–225.

[6] The alpha-tocopherol, beta-carotene cancer prevention study group (1994) The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *New England Journal of Medicine*, **330**, 1029–1035.

[7] G.S. Omenn, G.E. Goodman, M.D. Thornquist, J. Balmes, M.R. Cullen, A. Glass, J.P. Keogh, F.L. Meyskens, Jr., B. Valanis, J.H. Williams, Jr., S. Barnhart and S. Hammar (1996) Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *New England Journal of Medicine*, **334**, 1150–1155.

[8] C.H. Hennekens, J.E. Buring, J.E. Manson, M. Stampfer, B. Rosner, N.R. Cook, C. Belanger, F. LaMotte, J.M. Gaziano, P.M. Ridker, W. Willet and R. Peto (1996) Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *New England Journal of Medicine*, **334**, 1145–1149.

[9] W.A. Pryor, W. Stahl and C.L. Rock (2000) Beta-carotene: from biochemistry to clinical trials. *Nutrition Reviews*, **58**, 39–53.

[10] X.D. Wang and R.M. Russell (1999) Procarcinogenic and anticarcinogenic effects of β-carotene. *Nutrition Reviews*, **57**, 263–272.

[11] C. Wheatley (1997) Vitamin trials and cancer. *The Lancet*, **349**, 1844–1845.

[12] D.A. Cooper, A.L. Eldridge and J.C. Peters (1999) Dietary carotenoids and lung cancer: a review of recent research. *Nutrition Reviews*, **57**, 133–145.

[13] J.A. Mares-Perlman (1999) Too soon for lutein supplements. *American Journal of Clinical Nutrition*, **129**, 5–8.

[14] S.T. Mayne, G.J. Handelman and G.R. Beecher (1996) β-Carotene and lung cancer promotion in heavy smokers – a plausible relationship? *Journal of National Cancer Institute*, **88**, 1513–1515.

[15] J.B. Blumberg (1995) Considerations of the scientific substantiation for antioxidant vitamins and β-carotene in disease prevention. *American Journal of clinical Nutrition*, **62**(Suppl.), 1521S–1526S.

[16] A.T. Diplock (1995) Safety of antioxidant vitamins and β-carotene. *American Journal of Clinical Nutrition*, **62**(Suppl.), 1510S–1516S.

[17] M. Paolini, G. Cantelli-Forti, P. Perocco, G.F. Pedulli, S.Z. Abdel-Rahman, M.S. Legator (1999) Co-carcinogenic effect of beta-carotene. *Nature*, **398**, 760–761.

[18] M.G. Salgo, R. Cueto, G.W. Winston and W.A. Pryor (1999) Beta carotene and its oxidation products have different effects on microsome mediated binding of benzo[a]pyrene to DNA. *Free Radical Biology & Medicine*, **26**, 162–173.

[19] S.-L. Yeh and M.-L. Hu (2000) Antioxidant and prooxidant effects of lycopene in comparison with β-carotene on oxidant-induced damage in Hs68 cells. *Journal of Nutritional Biochemistry*, **11**, 548–554.

[20] G.J. Handelman, F.J. van Kuijk, A. Chatterjee and N.I. Krinsky (1991) Characterization of products formed during the autoxidation of β-carotene. *Free Radical Biology & Medicine*, **10**, 427–437.

[21] A.A. Woodall, G. Britton and M.J. Jackson (1997) Carotenoids and protection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals:

relationship between carotenoid structure and protective ability. *Biochimica Biophysica Acta*, **1336**, 575–586.
[22] T. Mosmann (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation cytotoxicity assay. *Journal of Immunological Methods*, **65**, 55–63.
[23] R.C. Gupta (1984) Nonrandom binding of the carcinogen *N*-hydroxy-2-acetylaminofluorene to repetitive sequences of rat liver DNA *in vivo*. *Proceedings of the National Academy of Science USA*, **81**, 6943–6947.
[24] M.K. Shigenaga, J.-W. Park, K.C. Cundy, C.J. Gimeno and B.N. Ames (1990) *In vivo* oxidative DNA damage: measurement of 8-hydroxy-2′-deoxyguanosine in DNA and urine by high-performance liquid chromatography with electrochemical detection. *Methods in Enzymology*, **186**, 521–530.
[25] M.-L. Hu and M.K. Shih (1997) Ascorbic acid inhibits lipid peroxidation but enhances DNA damage in rat liver nuclei incubated with iron ions. *Free Radical Research*, **26**, 585–592.
[26] N.P. Singh, M.T. McCoy, R.R. Tice and E.L. Schneider (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*, **175**, 184–191.
[27] A.R. Collins, A.G. Ma and S.J. Duthie (1995) The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. *Mutation Research*, **336**, 69–77.
[28] M.O. Parat, M.J. Richard, S. Pollet, C. Hadjur, A. Favier and J.C. Beani (1997) Zinc and DNA fragmentation in keratinocyte apoptosis: its inhibitory effect in UVB irradiated cells. *Journal of Photochemistry and Photobiology B: Biology*, **37**, 101–106.
[29] M. Sano, P.A. Motchnik and A.L. Tappel (1986) Halogenated hydrocarbon and hydroperoxide-induced peroxidation in rat tissue slices. *Journal of Free Radical Biology & Medicine*, **2**, 41–48.
[30] C.P. Lebel, H. Ischiropoulos and S.C. Bondy (1992) Evaluation of the probe 2′,7′-dichlorofluorencein as an indicator of reactive oxygen species formation and oxidative stress. *Chemical Research in Toxicology*, **5**, 227–231.
[31] J.A. Royall and H. Ischiropoulos (1993) Evaluation of the 2′,7′-dichlorofluorescein and dihydrohodamine 123 as fluorescent probe for intracellular H_2O_2 in cultured endothelial cells. *Archives Biochemistry and Biophysics*, **302**, 348–355.
[32] H.U. Bergmeyer (1974) Lactate dehydrogenase assay with pyruvate and NADH. In *Methods of Enzymatic Analysis*, Vol. 2 (ed. H.U. Bergmeyer), Academic press, New York, pp. 574–579.
[33] E. Niki, N. Noguchi, H. Tsuchihashi and N. Gotoh (1995) Interaction among vitamin C, vitamin E, and β-carotene. *American Journal of Clinical Nutrition*, **62**, 1322S–1326S.
[34] A. Mortensen and L.H. Skibsted (1998) Reactivity of β-carotene towards peroxyl radicals studied by laser flash and steady-state photolysis. *FEBS Letters*, **426**, 392–396.
[35] T.G. Truscott (1996) β-carotene and disease: a suggested pro-oxidant and anti-oxidant mechanism and speculations concerning its role in cigarette smoking. *Journal of Photochemistry and Photobiology B: Biology*, **35**, 233–235.
[36] F. Bohm, R. Edge, D.J. McGarvey and T.G. Truscott (1998) β-Carotene with vitamins E and C offer synergistic cell protection against NO. *FEBS Letters*, **436**, 387–389.
[37] J.A. Buege and S.D. Aust (1978) Microsomal lipid peroxidation. *Methods in Enzymology*, **52**, 302–310.
[38] D.L. Baker, E.S. Krol, N. Jacobsen and D.C. Liebler (1999) Reactions of β-carotene with cigarette smoke oxidants. Identification of carotenoid oxidation products and evaluation of the prooxidant/antioxidant effect. *Chemistry and Research Toxicology*, **12**, 535–543.
[39] C. Marty and C. Berset (1990) Factors affecting the thermal degradation of all-trans-β-carotene. *Journal of Agricultural and Food Chemistry*, **38**, 1063–1067.
[40] G.M. Lowe, L.A. Booth, A.J. Young and R.F. Bilton (1999) Lycopene and beta-carotene protect against oxidative damage in HT29 cells at low concentrations but rapidly lose this capacity at higher doses. *Free Radical Research*, **30**, 141–151.
[41] J.E. Romanchik, E.H. Harrison and D.W. Morel (1997) Addition of lutin, lycopene, or β-carotene to LDL or serum *in vitro*: effects on carotenoid distribution, LDL composition, and LDL oxidation. *Journal of Nutritional Biochemistry*, **8**, 681–688.
[42] X.D. Wang, G.W. Tang, J.G. Fox, N.I. Krinsky and R.M. Russell (1991) Enzymatic conversion of beta-carotene into beta-apo-carotenals and retinoids by human, monkey, ferret, and rat tissues. *Archives of Biochemistry and Biophysics*, **285**, 8–16.
[43] A. Nagao, A. During, C. Hoshino, J. Terao and J.A. Olson (1996) Stoichiometric conversion of all-trans-beta-carotene to retinal by pig intestinal extract. *Archives of Biochemistry and Biophysics*, **328**, 57–63.