Carotenoids inhibit DNA synthesis in human aortic smooth muscle cells

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Abstract Quiescent, serum-starved human aortic smooth muscle cells were restimulated with 20% foetal calf serum in Dulbecco's modified Eagle medium, in the presence and absence of β-carotene, canthaxanthin, zeaxanthin, lycopene, lutein or β-cryptoxanthin, at final concentrations up to 23 μM. Concentration-dependent inhibition of DNA synthesis, measured by [methyl-^{3}H]thymidine incorporation, was observed for the carotenoids, except for canthaxanthin and lutein which had no effect. Lycopene was the most potent of the carotenoids tested. The results suggest that antiproliferative effects of dietary carotenoids might be of significance in vivo.

Key words: Carotenoid; Lycopene; β-Carotene; DNA synthesis; Smooth muscle cell (human aortic); Atherosclerosis

1. Introduction

Carotenoids are plant pigments absorbed from fruits and vegetables in the diet. Epidemiological studies have shown associations between diets rich in fruits and vegetables and reduced risks of cancer, cardiovascular disease, and other age-related disorders [1]. This apparent protection has generally been attributed to naturally-occurring antioxidant radical scavengers, including α-tocopherol and carotenoids.

It is emerging that carotenoids have other biological effects besides radical scavenging, including antineoplastic and antiproliferative effects on a variety of cell lines [2–6]. Proliferation of smooth muscle cells (SMC) plays a major role in the alteration of the arterial wall in atherosclerosis [7–9]. Inhibition of SMC proliferation could be a mechanism by which carotenoids might affect progression of atherosclerosis.

The biological activities of the carotenoids are determined by their diverse molecular structures [10]. We therefore tested the main naturally-occurring dietary carotenoids, β-carotene, β-cryptoxanthin, zeaxanthin, lutein and lycopene, as well as canthaxanthin, which occurs in the edible mushroom *Cantharellus cinnabarinus* and is a permissible food additive, for their effects on DNA synthesis in human aortic SMC.

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Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiolazine-6-sulphonate); AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); DMEM, Dulbecco's modified Eagle medium; DNA, deoxyribonucleic acid; FCS, foetal calf serum; HMM, human monocyte macrophage; HPLC, high pressure liquid chromatography; LDL, low-density lipoprotein; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO_2, nitrogen dioxide; PBS, phosphate-buffered saline; PKC, protein kinase C; SMC, smooth muscle cells; TBARS, thiobarbituric acid-reactive substances; THF, tetrahydrofuran; v/v, volume/volume

2. Materials and methods

β-Carotene, canthaxanthin, zeaxanthin, lycopene, lutein and β-cryptoxanthin were gifts of Hoffmann-La Roche (Basel, Switzerland). Storage conditions and verification of purity (by HPLC and mass spectrometry) were as described previously [11]. Tetrahydrofuran (THF; HPLC grade) was obtained from Aldrich (Poole, Dorset, UK), [methyl-^{3}H]thymidine (88.7 Ci/mmol) from NEN (Hounslow, Middx., UK), and [2,8-^{3}H]adenine (24 Ci/mmol) from Amersham (Aylesbury, Bucks., UK). 24-Well tissue culture plates were from Falcon (Becton Dickinson). All other chemicals were obtained from Sigma (Poole, Dorset, UK). All chemicals were of the highest purity available.

Human aortic SMC, from Clonetics Corporation (TCS Biologicals Ltd., Buckingham, UK) at passage 3, were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% heat-inactivated foetal calf serum (FCS) (Life Technologies, Paisley, Scotland, UK), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells from passages 5–10 were used for experiments. Cells were plated at 10 000 cells/cm^2, i.e. 20 000 cells/well in 24-well tissue culture plates, with 1 ml culture medium per well. Prior to proliferation studies these subconfluent cells were rendered quiescent by partially depriving them of serum. This was done by removing the medium, washing the cells in the wells with PBS, and then replacing the medium with DMEM containing 0.2% FCS, for 48 h prior to the start of the proliferation experiment.

After the above 48 h serum deprivation period, the medium was removed and the quiescent SMC in the wells were restimulated to proliferate by re-exposing them to DMEM plus 20% FCS, with the addition of [methyl-^{3}H]thymidine (1 μCi/well), in the presence or absence of carotenoid, for 24 h. Carotenoid was added to the culture medium in THF (0.5% v/v final concentration) at the same time as [methyl-^{3}H]thymidine and FCS. This is an established method of adding carotenoids to cell cultures in a form available for cell uptake [11,12]. Carotenoids were tested at final concentrations up to 23 μM, with stock solutions freshly made up for each experiment. For incubations in the absence of carotenoid, THF (0.5% v/v) was added.

At the end of the 24 h restimulation period, the medium containing unincorporated radiolabel was removed, the cells in the wells were washed with phosphate-buffered saline (PBS), fixed with methanol, treated with 10% trichloroacetic acid, and digested with 0.2 M NaOH [13]. The radioactivity in the digests was measured by liquid scintillation counting [13].

Each experiment was repeated at least twice, and each frame of Fig. 1 (a–d) presents data from a representative experiment. Each data point represents the mean of triplicate wells; error bars denote standard error. Student's *t*-test (unpaired) was used to compare [methyl-^{3}H]thymidine incorporation in carotenoid-treated cells with that of control cells treated with THF vehicle (0.5% v/v), and to compare cells treated with β-carotene with cells treated with other carotenoids. Student's *t*-test was carried out using SigmaPlot 3.0 software.

The effect of carotenoids on proliferation of human aortic SMC was also tested in selected cases by measuring the reduction of the yellow, water-soluble dye 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide (MTT) to the dark blue insoluble formazan, as a result of the cells' dehydrogenase enzymes. The formazan is solubilised and the absorbance value so obtained is thus a measure of the total number of live cells in a culture. This well-established proliferation assay is based on the method of Mosmann [14]. Proliferation experiments were carried out as described above, except that [methyl-^{3}H]thymidine was omitted. As before, each treatment was performed in triplicate wells. At the end of the 24 h restimulation period, MTT dye (0.5 mg/ml final concentration) was added to the wells and the cells incubated for a further 3 h (at 37°C). The medium was removed and the insoluble formazan was solubilised in dimethylsulphoxide (200 μl per

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well) and transferred to a 96-well flat-bottomed plate. The absorbance was read at 570 nm, with background subtraction at 630 nm, using a Dynatech MR5000 plate reader. Results are presented in Table 1.

Thiobarbituric acid-reactive substances (TBARS) were measured in the medium, and in the cells, from SMC cultured as above in the presence and absence of carotenoids. The TBARS assay was carried out on the medium as described previously, with measurement of absorbance at 532 nm [15]. TBARS in the SMC were measured by a sensitive, fluorometric TBARS assay, adapted from that of Zhang and colleagues [3]. Briefly, the cells were scraped, treated with 1 ml of 0.08 N H_2SO_4 and 125 μl of 10% (w/v) phosphotungstic acid. The cells were centrifuged at 15000×*g* for 5 min, the supernatant was discarded, and the cells resuspended in 500 μl water. Thiobarbituric acid and trichloroacetic acid were then added and the rest of the processing carried out as described [15], except that the TBARS were finally extracted into 1 ml butanol, and the fluorescence read at 553 nm, with excitation at 515 nm [3]. Measurements were made on triplicate wells. TBARS concentrations were assessed as malondialdehyde (MDA) equivalents using a standard curve of 1,1,3,3-tetramethoxypropane.

3. Results and discussion

β-Carotene inhibited DNA synthesis in SMC, in a concentration-dependent fashion, as judged by incorporation of [methyl-^{3}H]thymidine (Fig. 1). These cells had been quiescence-synchronised by serum deprivation, verified by the established technique of flow cytometry with propidium iodide staining [16], prior to restimulation with FCS in the presence or absence of carotenoids. As well as inhibiting DNA synthesis under these quiescence-restimulation conditions, β-carotene was also effective at inhibiting DNA synthesis in SMC which had not been initially rendered quiescent (data not shown). Under the conditions used, we found no quenching of scintillation counts by carotenoids, so this could not explain apparent decreases in [methyl-^{3}H]thymidine incorporation.

The inhibition of DNA synthesis at these β-carotene concentrations was not due to toxicity of β-carotene. This was established using a panel of well-established toxicity assays: [^{3}H]adenine leakage [15], trypan blue dye exclusion, and fluorescein diacetate/propidium iodide dual staining measured by flow cytometry [17]. However, higher concentrations of β-carotene (47 μM) produced modest toxicity to SMC. The other carotenoids were not toxic at concentrations up to 23 μM. The THF vehicle (0.5% v/v) was non-toxic.

Canthaxanthin, tested up to 23 μM, was ineffective at inhibiting DNA synthesis ([methyl-^{3}H]thymidine incorporation) in SMC which were initially rendered quiescent by serum deprivation then restimulated with FCS in the presence and absence of carotenoids (Fig. 1a). β-Carotene, tested in the same experiment, inhibited DNA synthesis (Fig. 1a). Zeaxanthin, tested under the same conditions, in a separate experi-

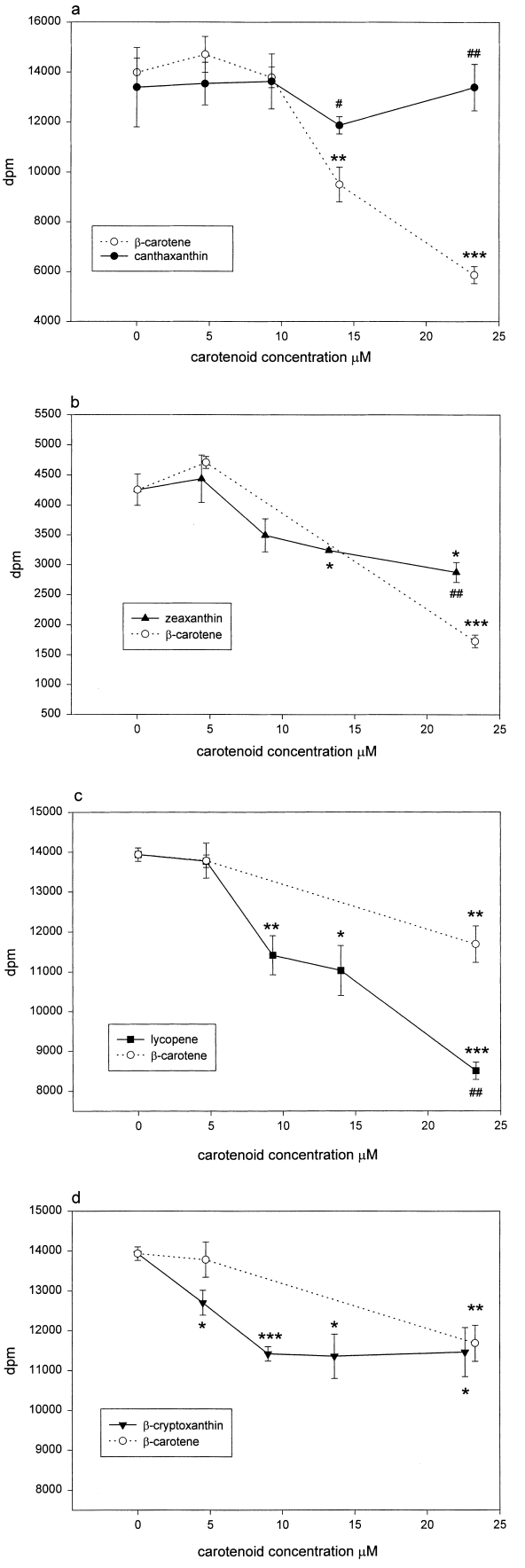

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Fig. 1. Effect of carotenoids on [methyl-^{3}H]thymidine incorporation in human aortic SMC. (a) β-Carotene (open circles) or canthaxanthin (filled circles), tested in parallel; (b) β-carotene (open circles) or zeaxanthin (filled triangles), tested in parallel; (c) β-carotene (open circles) or lycopene (filled squares), tested in parallel; (d) β-carotene (open circles) or β-cryptoxanthin (filled, inverted triangles), tested in parallel. Each data point represents the mean of triplicate wells; bars denote standard errors. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, carotenoid significantly different from control (no carotenoid, THF vehicle 0.5% v/v) by unpaired Student's *t*-test. $^{\#}P<0.05$, $^{\#\#}P<0.01$, carotenoid significantly different from β-carotene by unpaired Student's *t*-test. For further details see Section 2.

Table 1
Effect of carotenoids, tested in parallel, on proliferation of human aortic SMC, determined by measuring the reduction of MTT to formazan (absorbance at 570 nm, with background subtraction at 630 nm)

Addition	Concentration	*n*	Mean formazan absorbance	Standard error	*P*
THF vehicle	0.5% (v/v)	3	0.530	0.057	–
β-Carotene	4.7 μM	3	0.383	0.061	0.152
β-Carotene	23.3 μM	3	0.354	0.021	0.044
Lutein	4.7 μM	3	0.556	0.056	0.763
Lutein	23.3 μM	3	0.494	0.017	0.574
Lycopene	4.7 μM	3	0.352	0.013	0.038
Lycopene	23.3 μM	3	0.385	0.017	0.071

P values were obtained using Student's *t*-test (unpaired) to compare the formazan absorbance values in the presence of carotenoids with those of the THF vehicle control. For further details see Section 2.

ment, gave concentration-dependent inhibition of DNA synthesis, although it was not as potent as β-carotene, tested in parallel (Fig. 1b). The experiments described were repeated using the carotenoids lycopene, β-cryptoxanthin and lutein. Lycopene showed stronger, concentration-dependent inhibition than β-carotene when tested alongside (Fig. 1c). β-Cryptoxanthin inhibited DNA synthesis, with a similar potency at 23 μM to β-carotene in the same experiment (Fig. 1d). Lutein did not give significant inhibition of DNA synthesis (data not shown). Hydroxyurea (1 mM), used as a positive control in the above experiments, gave ca. 90% inhibition of DNA synthesis. None of the carotenoids was mitogenic for SMC under the conditions tested. Results of the MTT assay confirmed that β-carotene and lycopene were able to inhibit proliferation of human aortic SMC, and that lutein was ineffective (Table 1).

Possible mechanisms of the carotenoids' effects include inhibition of protein kinase C (PKC) activity, as reported for α-tocopherol in inhibiting proliferation of SMC in vitro [18,19]. PKC is important in the signal transduction pathway leading to cell proliferation and is a positive regulator in the transition of SMC from quiescence to proliferation [20]. Inhibition of proliferation might be linked to an antioxidant effect, or to formation of carotenoid products (e.g. epoxy-, peroxy- or hydroxy-derivatives) or metabolites (e.g. retinoids) in the cultures. These mechanisms may not be mutually exclusive. Carotenoids may also affect gene expression. For example, in C3H/10T1/2 cells, carotenoids increased gap-junctional communication by up-regulation of the *connexin-43* gene, apparently independently of the carotenoids' provitamin A properties or antioxidant effects [3,4]. In other examples, provitamin A properties of β-carotene appear to play a role [5], although this cannot explain the inhibition of DNA synthesis by lycopene or zeaxanthin, which are non-provitamin A carotenoids, in the present study. Another possible mechanism is that carotenoids might interfere with effects of growth factors [6].

Antioxidant effects of carotenoids could, in principle, be involved in inhibition of proliferation, since lipid peroxides, as well as oxidatively fragmented phospholipids and their enzymatic hydrolysis product lysophosphatidylcholine, are all mitogenic for SMC [21,22]. Recently, we demonstrated that β-carotene, canthaxanthin and zeaxanthin inhibited human monocyte macrophage (HMM)-mediated LDL oxidation in serum-free, iron-supplemented Ham's F-10 medium [11]. Under the same conditions, lycopene, lutein and β-cryptoxanthin were weaker and more variable in their antioxidant effects (unpublished own results). Whilst SMC can oxidise LDL under the same conditions as HMM [23], very little lipid peroxidation was evident in the present study, in which experiments were performed in DMEM plus 20% FCS, in either the supernatant medium or cells, in the absence or presence of carotenoids, as judged by measurement of TBARS (MDA≤0.25 nmol/ml of medium; MDA≤0.03 nmol per 20 000 cells). Similar values were obtained for control wells devoid of cells, containing the same medium in the presence and absence of carotenoids.

The order of potency, headed by lycopene, in the present study does not appear to reflect relative antioxidant potency of carotenoids against LDL oxidation [11], or against 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN)-induced oxidation of liposomes [24]. Lycopene was, however, the most potent of the common dietary carotenoids at scavenging the radical cation of 2,2′-azinobis(3-ethylbenzothiolazine-6-sulphonate) ($ABTS^{\bullet+}$) [25], and at quenching singlet oxygen [26], and although neither of these are likely to be directly relevant here, they may illustrate the idea that the open-chain lycopene might be more accessible to biologically active agents than the other common carotenoids which possess cyclised end groups. Also lycopene was more potent than β-carotene in several other situations. These included protection of lymphocytes from NO_2 radical-mediated damage [27], inhibition of DNA synthesis in MCF-7, Ishikawa and NCI-H226 cancer cell lines, attributed to intervention with signal transduction mechanisms of growth factors [6,28], and inhibition of cholesterol synthesis in the macrophage-like cell line J-774A.1 [29]. This last effect might also have antiproliferative consequences. Any of the above effects might contribute to an inverse correlation between carotenoids, especially lycopene, and atherosclerosis. The striking inverse correlations between lycopene and cancers [30–33], which are more significant than for other carotenoids [30–32], are consistent with the idea that lycopene might be the most important dietary carotenoid in terms of antiproliferative activity.

In the artery wall, proliferation of SMC gives rise to diffuse intimal thickening, as well as to fibrous cap formation in progressing atherosclerotic lesions. The latter may have a protective role in advanced atherosclerosis, as advanced lesions with thicker fibrous caps are less likely to rupture [34]. The formation of the fibrous cap occurs during the transition from fatty streak (early lesion) to intermediate lesion. In the very early stages of atherosclerosis, diffuse intimal thickening may provide the 'seedbed' for subsequent lesion formation. Carotenoids might play a part in inhibiting these various manifestations of SMC proliferation.

Our results are consistent with the inverse epidemiological association between dietary carotenoids and incidence of is-

chaemic heart disease [1]. Although carotenoids possess antioxidant activity, the contribution of this to the carotenoids' apparent protective effects has been questioned [35], and antiproliferative effects of carotenoids may be more important in vivo. The prepotency of lycopene in the present study is consistent with the apparently beneficial effect of 'Mediterranean' diets in this context, since tomatoes, the main food source of lycopene, are an abundant component of such diets.

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