β-Carotene Oxidation Products Modify Cholesterol Biosynthesis and Growth of Cultured Human Epidermoid Carcinoma Cell

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β-Carotene inhibits cholesterol production in A-431 cells *in vitro* and slightly modifies their growth in a culture. Oxidation of β-carotene increases its biological activity. The main products of β-carotene oxidation are isolated and their biological activity evaluated.

Key Words: *β-carotene; cholesterol production; apo-carotenals*

The risk of cardiovascular disease is lower in subjects using diets with a high content of carotenoids [2]. This has been explained by the antioxidant properties of carotenoids [7]. It was shown that β-carotene (BC) does not affect the resistance of low-density lipoproteins to oxidation but markedly reduces vascular injury in rabbits with hypercholesterolemia [14]. Other mechanisms of hypocholesterolemic effect of carotenoids were elucidated in recent years. BC posttranscriptionally regulates the activity of 3-hydroxy-3-methylglutaryl-CoA-reductase [4,13]. 3-Hydroxy-3-methylglutaryl-CoA-reductase, the key enzyme of isoprenoid biosynthesis, determines the rate of cholesterol (CS) production in animal cells and of carotenoids in plant cells. Its activity is regulated by the feedback mechanism by sterol and nonsterol metabolites of mevalonate [3] acting at different levels.

Recent studies on macrophage cultures demonstrated the effects of BC and licopin on CS metabolism, which were similar to the effects of fluvastin, a CS production inhibitor [5]. The production of CS can be effectively controlled by regulating the activities of other enzymes involved in the production of steroids, for example, inhibition of the activity of 2,3-oxidosqualenecyclase [10].

Possessing unsaturated double bonds, carotenoids can react with light, oxygen, heat, etc. As a trap for singlet oxygen, BC easily reacts with peroxide radicals [8]. Epoxides and carotenals with different length of chain are BC oxides [6]. Some oxides are more stable than the initial BC [15]. The metabolism of carotenoid oxides in man is little known, and they attract attention of scientists as important diet components [11,12].

We studied the effects of BC and its oxides on the production of CS and growth of A-431 culture.

MATERIALS AND METHODS

Synthetic BC (Hoffmann-La Roche) after recrystallization, CS (Sigma), radical initiator of azo-bis-isobutyronitrile (Sigma), potassium borohydride (KBH_4, Sigma), chromatographically pure solvents (Lekbiofarm) for high performance liquid chromatography (HPLC), and fresh-distilled tetrahydrofurane (THF) for cell culture were used.

Autooxidation of BC (2 mg/ml, 3.7 mM) was carried out in a chloroform solution at 37°C for 7 days, oxidation with azo-bis-isobutyronitrile (0.05 mg/ml, 0.03 mM) in a chloroform solution at room temperature for 3 days.

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TABLE 1. Biological Activity of BC preparations

Preparation	Concentration, μg/ml	CS production, %	Proliferation, %
Colloid dispersion of BC stabilized with proxanol-286	10	50	96
	5	63	96
	1	82	98
BC solution in THF	10	76	88
	5	87	94
	1	93	98
BC autooxidation products	10	47	92
	5	60	96
	1	78	100

Oxidized mixture was separated on a preparative 1.5×10 cm column with Kieselgel-60H (Merck): mobile phase chloroform, rate of elution 0.5 ml/min. By sight, 3 fractions were obtained, 7, 4.2, and 5.3 ml.

Fractions were separated by HPLC using Gilson-802 C pump and Gilson-116 spectrophotometer. Reverse-phase HPLC was carried out in a Zorbax ODS column (4.6×15 cm) in an isocratic system acetonitrile:methanol:isopropanol (60:10:30) [6]. Direct-phase HPLC was carried out in an isocratic system (5% ethyl acetate in hexane) at elution rate 1 ml/min. The spectra of resultant fractions were measured in a hexane solution in a Specord-M40 spectrophotometer.

The carbonyl groups were detected by the KBH_4 reduction test [6].

For investigation of biological activity of BC and its derivatives, the cells were cultured as described previously [1]. BC dispersion was stabilized with proxanol-286 and analyzed substances were added to cell culture [1]. The concentration of BC in the medium was no higher than 2×10^{-3} M. At higher concentrations the preparation is precipitated when added to the culture medium. If the concentration was higher, carotenoid solution in THF was very rapidly added to the culture medium on a magnetic stirrer under sterile conditions. After 18 h of incubation, ^{14}C acetate (3.3 μCi/ml) was added and incubated for 5 h. After lipid extraction with a hexane:isopropanol mixture (3:2), the monolayers were dissolved in 0.1 NaOH and an aliquot was taken for protein measurement [9]. The extract was evaporated, and CS was isolated by thin-layer chromatography on Kieselgel-60 F-254 plates (Merck) in hexane:diethyl ether:glacial acetic acid system (7:3:0.1). The CS radioactivity was measured in a liquid scintillator SL-30 (Intertechnique).

RESULTS

The biological activity test for BC and its derivatives *in vitro* included assessment of the effects of these compounds on the production of CS (by incorporation of ^{14}C acetate in cpm./mg cellular protein) and on the proliferative activity of cell culture (by milligrams of protein/dish). The results are the mean values of 3 experiments. Table 1 presents the biological activity of BC tested using different methods of its addition to the culture medium. THF is one of the best carotenoid solvents exerting no toxic effect on A-431 cells in the final concentration of up to 0.5%. BC concentration was measured by spectrophotometry using an extinction coefficient of 2550 for λ=451 nm. The final concentration of BC autooxidation products in the incubation medium

TABLE 2. Effect of Reduction on Biological Activity of BC Oxides

Preparation	Concentration, μg/ml	CS production, %	Proliferation, %
Third fraction	13	33	92
	3	59	98
	0.6	78	100
Third fraction, reduced	13	104	92
	3	106	98

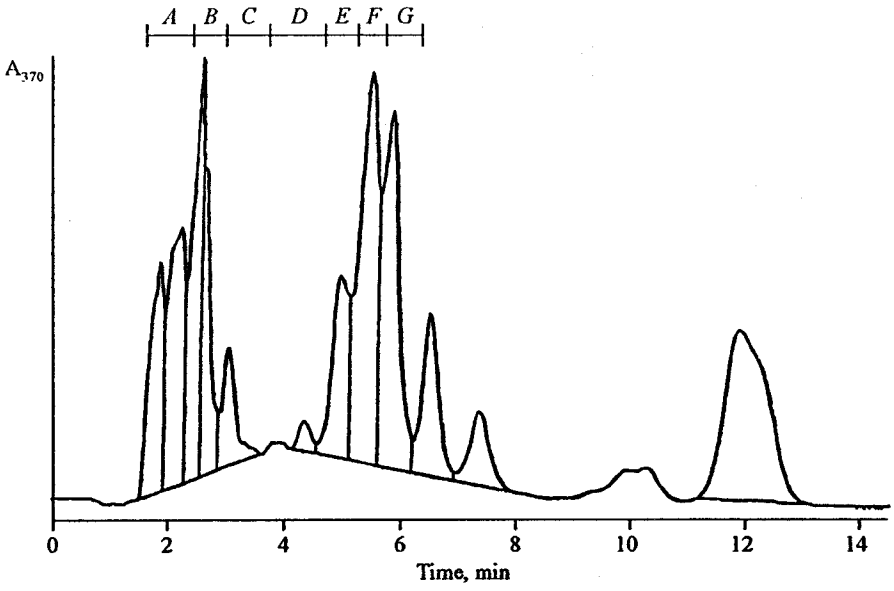

Fig. 1. Analysis of β-carotene oxidation products by high performance liquid chromatography. *A-G*: fractions collected.

was estimated from the content of BC in the initial solution subjected to oxidation.

Oxidation of BC leads to an appreciable decrease in the production of CS (Table 1). The proliferative activity of cultured cells changed negligibly. Autooxidation, as well as colloid dispersion of BC, a procedure requiring heating to 70°C, results in the formation of active oxidized products, which was confirmed by alteration of the absorption spectrum (data not presented) and by the loss of biological activity of oxidized derivatives of BC due to reduction. The results of reduction of the most active third fraction obtained by preparative chromatography of oxidized mixture are shown in Table 2.

TABLE 3. Biological Activity of BC Oxides after Fractionation

Fraction No.	λ_{max}, nm	CS production, %	Proliferation, %
A	285, 320	26	65
B	355, 375, 395	44	93
C	(400), 425	54	93
D	375, 400, 425	41	93
E	(425), 450	82	100
F	375, 395	65	100
G	(360), 375	71	88

Note. Here and in Table 4: weak maximum is given in parentheses.

TABLE 4. Biological Activity and Physicochemical Characteristics of BC Oxides

Peak No.	Time of release, min	λ_{max}, nm	CS production, %	Proliferation, %
1	4.3	(355), 380, 400	78	98
3	5.7	414	45	97
4	6.5	(384), 405, 428	35	91
5	6.9	333	65	94
6	7.5	375, 395	45	98
7	8.6	435	41	98
8	10.2	405, 425, 445	60	95

Note. Fraction No. 2 was not examined because of insufficient amount of material.

For elucidating the mechanism of inhibition of CS production by oxidized BC we attempted to detect the most active BC oxides in the mixture by a reverse-phase HPLC. Analysis of chromatograms showed that oxidation of BC results in the formation of a complex mixture of products. Seven fractions were isolated from it (*A-G*), characterized spectrophotometrically, and tested for biological activity (Table 3). The final concentration of the fractions was 1 μg/ml, which was determined by light absorbence of the relevant fractions. The extinction coefficient was taken for 2200 for the maximum absorption (λ_{max}) as the mean coefficient for apo-carotenals. All fractions added to cell culture were dissolved in THF.

Fractions *B*, *C*, and *D* had the most potent effect on CS production and negligibly modified cell proliferation in the culture (Table 3). Reverse-phase separation failed to show the peaks of desired resolution quality; therefore, for isolating individual substances additional purification by means of HPLC on silicagel was carried out. Fractions B, C, and D were united, evaporated, and layered in hexane onto a Zorbax Sil column (Fig. 2). Characteristics and biological activities of purified compounds are presen-

ted in Table 4. The final concentrations of substances in the incubation medium was measured by spectrophotometry and was 0.15 μg/ml. Group >C=O was present in all compounds.

Comparison of the data in Table 4 with the known characteristics of BC oxidation products [6,11,12] suggests that the isolated compounds are a series of carbonyls with different chain length: 1) unknown carbonyl, 3) apo-12'-carotenal, 5) apo-13-carotenone, 6) apo-14'-carotenal and retinal together, and 7) apo-10'carotenal. The shape of the absorption spectra peaks of Nos. 4 and 8 (with three maximums) suggests that these compounds contain an apo-aldehyde group and an epoxy group in the β-ring. Further studies are needed for detecting the structure of these compounds.

Our results indicate that all isolated carotenals inhibit the production of CS and negligibly affect cell proliferation in a culture. These findings may be useful for elucidating the mechanism of carotenoid effect on lipid metabolism in cells and for detecting new antihypercholesterolemic agents.

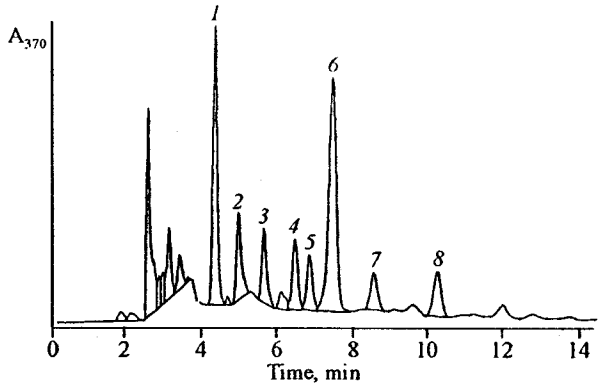

Fig. 2. High performance liquid chromatography on silicagel of pooled fractions *B*, *C*, and *D*. 1-8: peaks.

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