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Hypocholesterolemic Effect of Lycopene and β-Carotene Is Related to Suppression of Cholesterol Synthesis and Augmentation of LDL Receptor Activity in Macrophages

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β-Carotene and lycopene are derived from plants, and they share similar initial synthetic pathway with cholesterol, which is synthesized in animal but not in plant cells. Thus, we sought to analyze the effect of carotenoids on macrophage cholesterol metabolism, in comparison to the effect of LDL cholesterol and of the cholesterol synthesis inhibitor, fluvastatin. In J-774 A. 1 macrophage cell line, the cellular cholesterol synthesis from [^{3}H]-acetate, but not from [^{14}C] mevalonate, was suppressed by 63% and by 73% following cell incubation with β-carotene or lycopene (10μM) respectively, in comparison to a 90% and 91% inhibition by LDL (100μg of cholesterol), or by fluvastatin (10μg/ml) respectively. However, unlike LDL derived cholesterol, which also suppresses macrophage LDL receptor activity, lycopene and β-carotene augmented the activity of the macrophage LDL receptor, similarly to the effect of fluvastatin. In agreement with these in vitro observations, dietary supplementation of tomato's lycopene (60mg/day) to 6 males for a 3 months period resulted in a significant 14% reduction in their plasma LDL cholesterol concentrations. We thus conclude that dietary supplementation of carotenoids may act as moderate hypocholesterolemic agents, secondary to their inhibitory effect on macrophage 3-hydroxy-3-methyl glutaryl coenzyme A (HMGCoA) reductase, the rate limiting enzyme in cholesterol synthesis.

Cholesterol, an essential component of all animal cell membranes, is derived from the uptake of plasma low density lipoprotein (LDL) via receptor-mediated endocytosis (1), as well as from de novo cellular synthesis (2).

Over accumulation of cholesterol in cells is avoided by maintaining a balance between these external and internal cholesterol sources. This balance is achieved through feedback regulation on the biosynthetic pathway, and also on the LDL receptor synthesis. The enzyme 3-hydroxy-3 methylglutaryl coenzyme A (HMGCoA) reductase catalyzes the primary rate limiting step in the mevalonate biosynthetic pathway (3). Cholesterol, an end product of this pathway, negatively regulates the activity of HMGCoA reductase, and thus inhibits cellular cholesterol synthesis (3). In addition, cholesterol also inhibits the LDL receptor synthesis by repressing the LDL-receptor gene transcription (4). On the other hand, cellular cholesterol synthesis can also be inhibited by synthetic compounds such as the statins, which competitively inhibit the enzyme HMGCoA reductase. HMGCoA reductase inhibition by simvastatin was shown to trigger a coordinate regulation of the expression of the genes coding for the reductase and for the LDL receptor (5). In response to statins, the activity of the cellular LDL receptor is markedly increased in order to meet the cellular needs for cholesterol, resulting in an increased uptake of serum lipoprotein (6). Mevalonate is the precursor of a complex series of isoprenoids which are processed in animal cells not only into cholesterol, but also into other end products, such as dolichol, ubiquinon, and haeme (7). The activity of HMGCoA reductase in animal cells was shown to be sensitive to negative regulation by both sterols and non-sterol products of the mevalonate pathway (2,6). β-Carotene, and its precursor lycopene, are polyisoprenoids synthesized in plants from mevalonate via the HMGCoA reductase pathway. In plants, as in animal cells, HMGCoA reductase activity is regulated by an end product repression (8). A recent study have demonstrated that β-carotene regulates the expression of HMGCoA reductase in rat liver by a post-transcriptional mechanism (9). Based on the concept

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Abbreviations: LDL, low density lipoprotein; HMGCoA, hydroxy methyl glutaryl coenzyme A.

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that excess of any end product in the mevalonate pathway could regulate the production of other products of the same biosynthetic pathway, we have studied the effect of lycopene and of β-carotene on cholesterol metabolism in macrophages. Our results clearly demonstrate that macrophage enrichment with lycopene or with β-carotene results in the suppression of cellular cholesterol synthesis and increased macrophage LDL receptor activity. This effect can lead to enhanced clearance of LDL from the plasma, and thus carotenoids may be recognized as hypocholesterolemic agents.

METHODS

Materials. Tomato's lycopene was supplied by LycoRed, Natural Products Industries, Ltd, Beer Sheva, Israel. Synthetic β-carotene was obtained from Hoffman-LaRoche (Basel, Switzerland). Fluvastatin was obtained from Sandoz Pharma Ltd., (Basel, Switzerland). [^{3}H]acetate sodium salt and [^{14}C]mevalonol-actone were purchased from Amersham (Amersham International, Bucks, UK). Tissue culture media Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin and L-glutamine were obtained from Beth-Haemek, Biological Industries, Israel, and bovine serum albumin (BSA) was from Sigma Chemicals (St. Louis, MO).

Cells. J-774 A.1 macrophage-like cell line was obtained from the American Tissue Culture Collection (ATTC, Rockville, MD), and was maintained in DMEM containing 100U/ml penicillin, 100μg/ml streptomycin and 5% heat inactivated (56°C for 30 min) FCS.

Lipoproteins. Human LDL was separated from venous blood drawn into 1mg/ml of disodium EDTA, by discontinuous density gradient ultracentrifugation at d=1.019-1.063 g/ml (10). LDL was radioiodinated by the iodine monochloride method as modified for lipoproteins (11).

Carotenoids analyses. The content of β carotene and lycopene in macrophages was determined by high performance liquid chromatography (HPLC).

Incubation procedure. Macrophages were preincubated for 48 hours with DMEM supplemented with 10% FCS, to which lycopene or β-carotene dissolved in tetrahydrofuran (THF) were added. Control cells were incubated with similar volume of THF, and the THF volume added did not exceed 1%. For cell enrichment with cholesterol, the cells were preincubated for 48 hours with DMEM containing 100μg of LDL cholesterol/ml. Similarly, fluvastatin was added directly to the culture medium and incubated with the cells for 48 hours.

Cholesterol synthesis. Cellular cholesterol biosynthesis was assayed by incubation of the cells for 18 hours with [^{3}H]acetate (1μCi/ml), or with [^{14}C]mevalonolactone (1μCi/ml), after which cellular lipids were extracted in hexane: isopropanol (3:2, v:v), separated by thin layer chromatography (TLC) on silica gel plates, and developed in hexane:ether: acetic acid (80:20:1.5, v:v:v). Unesterified cholesterol spots were visualized by iodine vapour (using appropriate standard) scraped into scintillation vials and counted in a β-counter.

Cellular uptake of LDL. Macrophages were incubated for 5 hours at 37°C with 10μg of [^{125}I]LDL protein/ml. LDL cellular degradation was measured in the collected medium as the trichloroacetic acid (TCA) soluble, non-lipid, non iodide radioactivity (12). Lipoprotein degradation in a cell free system, which was measured under identical conditions, was less than 10% and was always subtracted from the total degradation. The remaining cells were washed 3 times with cold PBS and dissolved in 0.1N NaOH for measurement of cell-associated [^{125}I]LDL. An aliquot of these cells was taken for protein determination by the method of Lowry et al (13), using bovine serum albumin (BSA) as a standard.

TABLE 1

Macrophae Enrichment with Carotenoids

Concentration added (μM)	Cellular carotenoid concentration (nmol/mg cell protein)	
	Lycopene	β-Carotene
0.0	0.0	0.0
2.5	0.7 ± 0.2	0.4 ± 0.2
5.0	1.6 ± 0.3	0.8 ± 0.2
10.0	3.6 ± 0.6	1.5 ± 0.3

Note. J-774 A.1 macrophages were incubated for 48 hours at 37°C with DMEM supplemented wtih 5% FCS, in the presence of increasing carotenoid concentrations. Then, cellular lipids were extracted and the carotenoids content was measured by HPLC. Results are expresses as mean ± S.D. (n = 3).

In vivo studies. Six healthy males, age 30-35, under no medication, were administered 60mg of tomato's lycopene/day for a period of 3 months. Blood samples were drawn before, and 3 months after lycopene consumption. LDL cholesterol was determined by an enzymatic kit (Raichem, Ltd).

Statistical analysis. All experiments were performed in triplicates. Results are expressed as mean ± S.D. (n=3) Student's t test was performed for the statistical analyses.

RESULTS

Upon incubation of J-774 A.1 macrophages with increasing concentrations of lycopene or with β-carotene, cell enrichment with these carotenoids, in a dose-dependent manner was demonstrated (Table 1). However, at similar added concentrations, lycopene was taken up into the cells about two fold more than β-carotene. The effect of carotenoids on cellular cholesterol synthesis from [^{3}H]acetate was studied and compared to the inhibition of cellular cholesterol synthesis in cholesterol-enriched cells and in cells that were treated with statins. Macrophages were preincubated for 48 hours with increasing concentrations of LDL or of fluvastatin followed by incubation with [^{3}H]acetate for 18 hours at 37°C. [^{3}H]acetate incorporation into newly synthesized cholesterol was inhibited in a dose-dependent manner, reaching a maximum inhibition of 88% and 98% on using 60μg of LDL cholesterol/ml, or 1μg of fluvastatin/ml, respectively (Fig 1). Similarly, supplementation of the cells with increasing concentrations of β-carotene or lycopene resulted in a dose-dependent inhibition of macrophage cholesterol synthesis from [^{3}H]acetate. However, lycopene, when added at a similar concentration as β-carotene, inhibited cellular cholesterol synthesis more effectively than β-carotene along all studied concentrations. On using 10μM of lycopene or β-carotene cholesterol synthesis was inhibited by 73% or by 63%, respectively (Fig 1). Both choles-

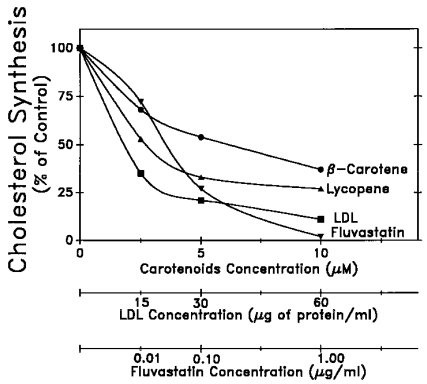

FIG. 1. The effect of carotenoids, LDL and fluvastatin on cellular cholesterol synthesis in J-774 A. 1 macrophages. Cells were incubated with increasing concentrations of β-carotene, lycopene, LDL, or fluvastatin for 48 hours at 37°C, following by the addition of [^{3}H]-acetate (1μCi/ml) and a further incubation for 18 hours at 37°C, prior to analyses of cellular [^{3}H]labeled cholesterol. Cells incubated with no additions were used as control. Results are given as percentage of the control value and represent one out of 3 experiments with similar results.

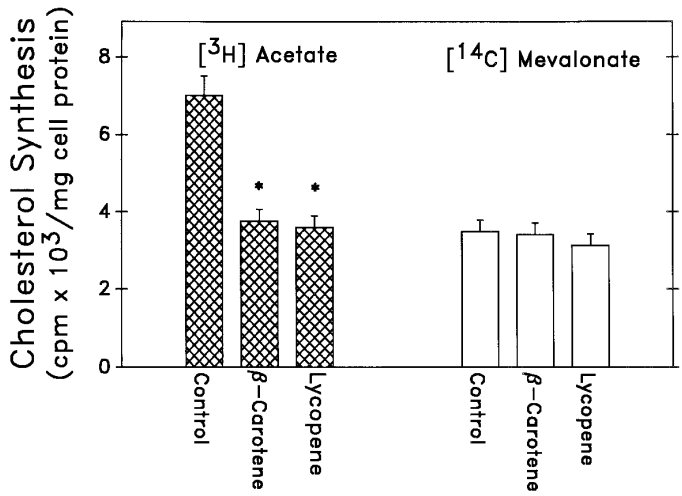

FIG. 2. The effect of β-carotene or lycopene on macrophage cholesterol synthesis from [^{3}H]acetate (hatched bars) or from [^{14}C]-mevalonate (open bars). The carotenoids were used at a concentration of 10μM. Results are given as mean ± S.D. (n=3).

terol and fluvastatin inhibit cellular cholesterol synthesis as a result of their inhibitory effect on the enzyme HMGCoA reductase, though by different mechanisms. In order to examine whether β-carotene or lycopene also affect cholesterol synthesis along this pathway from acetyl CoA to mevalonate, we tested the ability of the carotenoids to inhibit cholesterol synthesis from [^{14}C]mevalonate. Incubation of J-774 A.1 macrophages with 10μM of β-carotene or lycopene under conditions that suppressed [^{3}H]acetate incorporation into cellular cholesterol by ~40% did not result in any significant inhibition in cholesterol synthesis from the radiolabeled mevalonate (Fig 2). These data strongly suggest that β-carotene and lycopene regulate macrophage cholesterol synthesis by inhibiting cellular HMGCoA reductase activity. Inhibition of cholesterol synthesis by cellular cholesterol loading inhibits LDL receptor activity, whereas statins, which also inhibit cholesterol synthesis (by a different mechanism), stimulate the LDL receptor activity. We thus studied the effect of cells enrichment with carotenoids on macrophage LDL receptor activity, in comparison to the effects of LDL-cholesterol or statins. Fig 3 demonstrates that on using 10μM of carotenoids, macrophage enrichment with β-carotene or with lycopene resulted in 25% or 34% increased cellular LDL degradation (Fig 3A), and in 36% or 109% increased cell-association of LDL (Fig 3B), similarly to the effect of fluvastatin (1μg/ml) and in contrast to the inhibitory effect of 50μg of LDL cholesterol/ml (Fig 3). These results suggest that cell enrichment with carotenoids can increase the removal of LDL from the extracellular spaces, including the plasma environment. In order to test this possibility we administered 60mg of lycopene/day to 6 healthy males for a period of 3 months. This treatment resulted in a significant ($p<0.02$) 14% reduction in plasma LDL-cholesterol concentration (from 165±10 mg/dl before lycopene supplementation to 140±11 mg/dl after 3 months of lycopene consumption), with no effect on HDL-cholesterol concentrations (data not shown).

DISCUSSION

The present study demonstrates that β-carotene, and its precursor lycopene, suppress cellular cholesterol

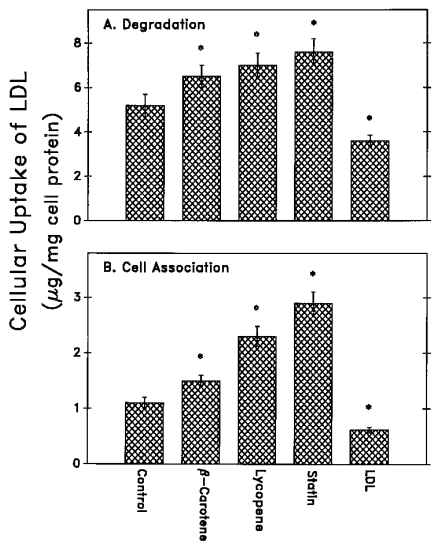

FIG. 3. The effect of β-carotene (10μM), lycopene (10μM), LDL (50μg of cholesterol/ml) or fluvastatin (1μg/ml), on macrophage LDL degradation (A) and cell-association (B). Results are given as mean ± S.D. (n=3).

synthesis from acetate, but not from mevalonate, in a concentration-dependent manner. This inhibition was concomitant with a stimulation of the LDL receptor activity in macrophages which could lead to enhanced clearance of LDL from the plasma. Indeed this was confirmed by the in vivo study, where a 14% reduction in plasma LDL cholesterol concentration was obtained in healthy males following lycopene administration.

We have previously shown that carotenoids can bind to lipoproteins (14,15) and to macrophages (16), and to affect their oxidative state. The increased potency of lycopene over β-carotene to inhibit cellular cholesterol synthesis and to increase macrophage LDL receptor activity may be related to the enhanced uptake of lycopene over β-carotene by macrophages (which may be related to their different structure and lipophylity).

Since the carotenoids (β-carotene and lycopene) which are present in plant cells, and cholesterol which is present in animal cells, share the same synthetic pathway, we compared their capacities to regulate cellular cholesterol synthesis in macrophages. It was already demonstrated that cellular cholesterol inhibits HMGCoA reductase activity by repression of the enzyme gene transcription through an effect on the sterol regulating element (SRE 1) (17).

Since the LDL receptor gene also contains the SRE 1 nucleotide sequence, it is also repressed by cholesterol, resulting in reduced LDL receptor synthesis and in reduced cellular uptake of LDL via the LDL receptor. Lycopene and β-carotene also suppressed macrophage cholesterol synthesis from [^{3}H]acetate, but not from [^{14}C]mevalonate, suggesting that these carotenoids may also inhibited cellular HMGCoA reductase. β-Carotene was indeed shown to inhibit the expression of HMGCoA reductase in rat liver by a post-transcriptional mechanism (9). HMGCoA reductase activity was already demonstrated to be regulated at the post-transcriptional level also by non-sterol metabolites of the mevalonate pathway (18). Plant foods were shown to possess cholesterol suppressive capacity, which was attributed to non-sterol post mevalonate products (19). The inhibition of HMGCoA reductase activity in mammalian cells by excess cholesterol is associated with reduced LDL receptor activity secondary to sterol-induced inhibition of the LDL receptor gene transcription (20). In carotenoids-enriched cells, no such cholesterol accumulation occurs, and therefore the LDL receptor synthesis is not inhibited. Indeed, our results demonstrated an increased LDL uptake by macrophages that were enriched with either lycopene or β-carotene, in contrast to the reduced uptake of LDL by cholesterol-enriched macrophages. In light of these results, the effect of the carotenoids on macrophage cholesterol metabolism was compared to that of the HMGCoA reductase inhibitors, the statins. Inhibitors of cholesterol biosynthesis are known to reduce serum cholesterol concentrations by enhancing the removal of serum LDL, secondary to activation of the LDL receptors (21). Thus, inhibition of HMGCoA reductase by carotenoids, similarly to the effect of the HMGCoA reductase inhibitor, fluvastatin, probably triggers a coordinate increased expression of the genes which are coding for the LDL receptor. In fact, similar effects were demonstrated by other plant-derived isoprenoids such as tocotrienols (22-24).

The minor but significant hypocholesterolemic effect of carotenoids on plasma LDL-cholesterol levels were evidenced in vivo after lycopene consumption by healthy volunteers for a 3 months period. Synthetic and natural tocotrienols, as well as other isoprenoids were already shown to possess hypocholesterolemic effects (24). Hypercholesterolemia is a major risk factor for atherosclerosis (25) and thus reduction of plasma cholesterol concentration by dietary consumption of carotenoids may reduce the risk cardiovascular diseases (25). Dietary supplementation of phytosterols to the atherosclerotic apolipoprotein E deficient mice was recently shown to reduce their plasma cholesterol concentration, and to inhibit the development of atherosclerosis (26). In another study, β-carotene administration together with a high-cholesterol diet to rabbits also inhibited the formation of atherosclerotic lesion (27).

Inhibition of cholesterol synthesis secondary to the inhibition of cellular HMGCoA reductase can be achieved by completely different agents and via different mechanisms; whereas cholesterol regulates the HMGCoA reductase gene transcription, statins are competitive inhibitors of this enzyme. Carotenoids, in contrast, inhibit HMGCoA reductase at a post-transcriptional level. Altogether these different mechanisms result in a similar inhibition of cellular cholesterol synthesis. However, while cholesterol concomitantly inhibits the transcription of the LDL receptor gene and reduces cellular uptake of LDL, carotenoids, similarly to statins, augment LDL receptor activity and can thus reduce plasma cholesterol concentration, hence, acting as hypcholesterolemic natural nutrients agents.

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