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Supplementation with β-carotene in vivo and in vitro does not inhibit low density lipoprotein oxidation

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Abstract

The inhibition of low density lipoprotein (LDL) oxidation has been postulated as one mechanism by which antioxidants may prevent the development of atherosclerosis. Available data on the ability of β-carotene to inhibit LDL oxidation are conflicting. We examined the role of in vivo and in vitro supplementation with β-carotene on metal ion-dependent (cupric ions, Cu^{2+}) and metal ion-independent (2,2′-azobis[2-amidinopropane]dihydrochloride, AAPH) oxidation of LDL as measured by the formation of conjugated dienes (absorbance at 234 nm). Sixteen subjects were supplemented with 50–100 mg of β-carotene on alternate days for 3 weeks following a week-long loading dose of 100 mg/day. Plasma β-carotene levels rose 5.5-fold, while LDL β-carotene levels rose 8.5-fold. Oxidation of LDL by Cu^{2+} or AAPH was not significantly delayed after in vivo supplementation with β-carotene compared with baseline. For AAPH, the lag phase (in minutes) was 75 ± 8 at baseline and 83 ± 14 after supplementation ($P = 0.07$). For Cu^{2+}, the lag phase was 172 ± 41 at baseline and decreased to 130 ± 24 after supplementation ($P < 0.01$). Similarly, no protective effect against Cu^{2+}-induced oxidation was observed when β-carotene was added to LDL in vitro. Supplementation of plasma with β-carotene in vitro prior to LDL isolation also did not enhance LDL's resistance to Cu^{2+}- or AAPH-induced oxidation, despite a 5-fold increase in LDL β-carotene levels over vehicle control. These data indicate that supplementation with β-carotene in vivo or in vitro does not enhance the protection of LDL against metal ion-dependent and -independent oxidation; rather, in vivo β-carotene supplementation may lead to a shortening of the lag phase of Cu^{2+}-induced lipid peroxidation in LDL.

Keywords: Antioxidants; β-Carotene; Carotenoids; LDL; Lipid peroxidation; Atherosclerosis

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1. Introduction

Recent evidence suggests that oxidative damage, particularly oxidative modification of LDL, is causally involved in the initiation and promotion of atherosclerotic disease [1]. The 'oxidation hypothesis of atherosclerosis' raises the possibility that antioxidants, such as β-carotene, may prevent or delay the progression of atherosclerosis. Several observational, prospective cohort studies have examined the role of β-carotene in heart disease, and all have shown a risk reduction associated with the ingestion of foods rich in β-carotene. For example, researchers with the Nurses' Health Study found that women in the highest quintile of β-carotene consumption had a 22% risk reduction for coronary disease (P-value for trend = 0.02) compared with women in the lowest quintile [2]. The Health Professionals Follow-up Study compared men in the highest quintile of intake of β-carotene with men in the lowest quintile, and the relative risk for major coronary events was 0.75 (P, trend = 0.04) [3]. In addition, a recent case-control study found that the risk of myocardial infarction was 78% higher for subjects in the lowest as compared to the highest quintile of adipose tissue β-carotene levels, after adjustment for age, body-mass index, and smoking [4].

Limited randomized trial data on the effect of β-carotene supplementation on atherosclerotic disease are conflicting. A subgroup analysis within the Physicians' Health Study of 333 doctors with a history of chronic stable angina or prior coronary revascularization procedure showed that among subjects who received β-carotene there was a 51% reduction in risk of major coronary events ($P < 0.05$), and a 54% reduction in risk of major vascular events ($P < 0.02$) [5]. Furthermore, the effect of β-carotene supplementation on clinical events was time-dependent [5], consistent with the theory that antioxidant intake slows the progression of atherosclerosis. In contrast, a recent study of long-time male smokers given daily supplements of 20 mg of β-carotene for 6 years did not find a protective effect against ischemic heart disease [6].

The specific mechanisms by which β-carotene may affect the development of cardiovascular disease are unclear. Both the in vivo and the in vitro properties of β-carotene have been reviewed recently [7]. β-Carotene, a pigment found in high concentrations in many yellow and orange fruits and vegetables and green leafy vegetables, has the ability to quench singlet oxygen [8] and can also act as a chain-breaking antioxidant under certain conditions [9]. β-Carotene is transported within circulating lipoproteins and has been postulated to inhibit the oxidative modification of LDL. Interestingly, reports on the ability of β-carotene supplementation in vitro to protect against LDL oxidation are conflicting [10–13]. The current data on the effects of β-carotene supplementation in vivo on Cu^{2+}-induced LDL oxidation ex vivo are more consistent and do not suggest significant protection [14,15]. In contrast, the effects of both in vivo and in vitro β-carotene supplementation on metal ion-independent oxidative modification of LDL are not known. This question is relevant, as it is currently unclear whether LDL oxidation in vivo occurs by a metal ion-dependent or -independent mechanism. Therefore, in the present study, we examined the impact of both in vivo and in vitro supplementation with β-carotene on metal ion-dependent and -independent oxidation of LDL.

2. Experimental procedures

2.1. In vivo supplementation with β-carotene

The study group consisted of 16 volunteers (12 women and 4 men), age 24–47 years (33 ± 8 years, mean ± S.D.), with a body-mass index of 22.5 ± 2.5 kg/m^2 (mean ± S.D.), and with no prior history of coronary heart disease, cancer, active liver disease, cirrhosis, atrophic gastritis, pancreatic disease, small bowel disease or resection, bleeding disorder, insulin-requiring diabetes, or renal disease. All study subjects were on average American diets, and agreed to abstain from supplementation with β-carotene, vitamin A, or vitamin E for the trial period. Seven subjects were current smokers, three past smokers, and six had never smoked.

Subjects were randomized into two groups of equal size receiving either natural β-carotene (Betatene, Henkel Corporation, Fine Chemicals Division, La Grange, IL) or synthetic β-carotene

(Lurotin — 10% β-carotene powder, BASF, Ludwigshafen, Germany). Natural β-carotene is isolated from the sea alga *Dunaliella salina*, and is composed of approximately equal proportions of the all-*trans* and 9-*cis* isomers of β-carotene, which together constitute >90% of carotenoids found in the natural preparation. Synthetic β-carotene has an all-*trans* isomeric configuration. Natural β-carotene was provided as gel caps containing 33 mg of β-carotene, while synthetic β-carotene was provided as capsules containing 50 mg in a water-stabilized preparation.

During the first 6 days of the study, subjects in both groups consumed 100 mg/day of β-carotene agent. After this loading period, subjects given synthetic β-carotene took a maintenance dose of 50 mg on alternate days for 3 weeks. The group receiving natural β-carotene was further randomized to receive either 66 or 100 mg on alternate days for 3 weeks. The maintenance dose was higher for the natural substance because its bioavailability is lower. The 9-*cis* β-carotene isomer represented only a minor fraction (<10%) of the total β-carotene present in plasma, both before and after the supplementation period. The increases in plasma β-carotene levels were essentially proportional to the all-*trans* β-carotene content of the supplement used. A detailed description of the bioavailability data will be published separately. For the purposes of the present study, it was sufficient to measure β-carotene levels in LDL and correlate them with parameters of LDL oxidation.

Blood samples were obtained at baseline, and at the end of the 30-day supplementation period. Subjects had been fasting, and samples were obtained between 07:30 and 09:30 h. The blood was drawn into heparin vacuum tubes and used immediately for LDL isolation and LDL β-carotene analysis. For plasma β-carotene determination, blood was drawn into EDTA vacuum tubes and plasma samples were frozen at −20°C until analysis.

2.2. *In vitro supplementation with β-carotene*

For these studies, blood was drawn into heparin vacuum tubes after an overnight fast from three healthy, normolipidemic subjects (one women and two men, age 27–39 years), who did not participate in the above in vivo β-carotene supplementation study. Two methods were used for in vitro supplementation: either β-carotene was added to plasma followed by isolation of LDL, or β-carotene was added to LDL which had been isolated from unsupplemented plasma. For the former method, 25 μl of a solution containing 21.5 mM synthetic β-carotene (Fluka, Buchs, Switzerland) in peroxide-free tetrahydrofuran (THF) was added per ml of plasma. After a 5 min incubation period at 37°C, non-lipoprotein-associated β-carotene was removed by gel filtration [16] and LDL was isolated from the plasma as described below. We found that LDL prepared in this way from β-carotene- or vehicle (THF)-treated plasma contained varying amounts of lipid hydroperoxides (as measured by high performance liquid chromatography (HPLC) with chemiluminescence detection [17]). Since pre-formed lipid hydroperoxides in LDL affect the lag phase of Cu^{2+}-induced lipid peroxidation [16,18], LDL isolated from treated plasma was incubated with the synthetic peroxidase Ebselen (Cayman Chemical Company, Ann Arbor, MI) and reduced glutathione (GSH) in order to reduce pre-formed lipid hydroperoxides to alcohols [19]. Ebselen and excess GSH were subsequently removed by sequential passage of the LDL preparation through two PD-10 columns (Pharmacia, Uppsala, Sweden) [19]. Alternatively to the above method of in vitro supplementation of plasma prior to LDL isolation, β-carotene in hexane or THF was added directly to LDL incubations (see legend to Table 2 for further details on this method).

2.3. *Isolation of LDL*

LDL was isolated as described in [16]. Briefly, plasma was obtained from heparinized blood by centrifugation at 4°C for 10 min at 1000 × *g*. To avoid interference with measurement of the lipid peroxidation lag phase by residual amounts of vitamin C and uric acid in the LDL preparations [20], these water-soluble antioxidants were removed from the plasma by gel filtration using a column centrifugation technique [16]. The density of the gel-filtered plasma was then adjusted to 1.21 g/ml, and LDL was isolated by single spin discontinuous density gradient ultracentrifugation at 7°C and

80 000 rev./min for 45 min, using a near vertical tube 90 rotor in an L8-80M ultracentrifuge (Beckman Instruments, Palo Alto, CA) [16]. The LDL fraction was removed from the tube by inserting a syringe needle directly into the LDL band and suctioning. Chelex resin (approximately 0.1 g/ml) was added to LDL to bind adventitious metal ions, thus minimizing fortuitous oxidation, and this preparation was filtered through a 0.2 μm syringe filter (Gelman Sciences, Ann Arbor, MI) to remove the resin. Protein concentration was determined by the method of Lowry, with sodium dodecylsulfate added to the assay buffer to facilitate dissolution of the lipoprotein [16]. Bovine serum albumin was used as standard. LDL was stored under nitrogen at 4°C in the dark and used within 2 h for incubations with Cu^{2+} or AAPH and β-carotene determination.

2.4. Measurement of plasma and LDL β-carotene concentrations

Plasma β-carotene concentrations were determined by reverse-phase HPLC with detection at 455 nm as described earlier by us [21]. β-Carotene levels in LDL were quantified by HPLC with electrochemical detection. A 200 μl sample containing 0.1 mg of LDL protein in 10 mM phosphate-buffered saline, pH 7.4 (PBS) was extracted with 200 μl of methanol and 2.5 ml of hexane. After centrifugation, 2 ml of the hexane phase was removed and evaporated to dryness under nitrogen, and the residue was dissolved in 240 μl of ethanol. A 50 μl aliquot was analyzed by reverse-phase HPLC using an LC-8 column (Supelco, Bellefonte, PA), and 1% water in methanol containing 20 mM lithium perchlorate as mobile phase. The eluate was analyzed by electrochemical detection at an applied potential of +0.6 V in an LC 4B amperometric electrochemical detector (Bioanalytical Systems, West Lafayette, IN). Both HPLC systems used for β-carotene measurements in plasma and LDL were calibrated daily using fresh standard solutions of synthetic β-carotene in hexane. The concentration of the standard solution was determined spectrophotometrically, with a molar extinction coefficient at 465 nm of $\epsilon_{465\ nm} = 1.29 \times 10^5/M$ and cm [22].

2.5. Oxidation of LDL

During the course of lipid peroxidation in LDL, conjugated dienes of polyunsaturated fatty acids are formed which have an absorbance maximum at 234 nm. Thus, oxidation of LDL can be followed by measuring the absorbance at this wavelength. The time course of absorbance change can be divided into three distinct phases: the lag, propagation, and decomposition phases [23]. During the lag phase, 234 nm absorbance increases slowly, indicating resistance of LDL to oxidation. Subsequently, absorbance increases rapidly during the propagation phase of lipid peroxidation. This is followed by the decomposition phase, during which the lipid hydroperoxides decompose to form aldehydic and an array of other products.

Thus, LDL in PBS (0.1 mg of protein/ml) was exposed to either 1.25 μM $CuCl_2$ or 4 mM AAPH and 0.1 mM diethylenetriaminepentaacetic acid (to chelate adventitious metal ions and prevent them from contributing to AAPH-induced LDL oxidation). AAPH is a water-soluble azo compound that thermally decomposes, leading to formation of aqueous peroxyl radicals at a constant rate [16]. Samples were incubated in a 6-cell holder in a spectrophotometer (U-2000, Hitachi, Japan) at 37°C. The reference cuvette contained either 1.25 μM $CuCl_2$ in PBS or 4 mM AAPH and 0.1 mM diethylenetriaminepentaacetic acid in PBS. Since AAPH strongly absorbs at 234 nm, the use of a proper reference solution was particularly important in this case. The absorbance at 234 nm was recorded every 10 min. The length of the lag phase of lipid peroxidation was determined as the intercept of the two tangents drawn to the lag and propagation phases of the 234 nm absorbance curve. The coefficients of variation for the length of the lag phase for Cu^{2+}- and AAPH-induced lipid peroxidation in LDL were 7.5% and 5.1%, respectively [16]. The rate of lipid peroxidation during the propagation phase in Cu^{2+}-exposed LDL was determined as the maximal slope of the absorbance curve, using a molar extinction coefficient for conjugated dienes of $\epsilon_{234nm} = 2.95 \times 10^4/M$ and cm [23]. The rate of lipid peroxidation during the propagation phase in LDL incubated with AAPH could not be determined as the absor-

bance at 234 nm reached maximal measurable values before the propagation phase was completed [16].

2.6. Statistical methods

For all variables, mean values and standard deviations are presented. The paired Student's *t*-test was used for comparison between pre- and post-supplementation samples.

3. Results

3.1. In vivo supplementation

Baseline characteristics and results obtained on LDL oxidizability for the three treatment groups (50 mg of synthetic β-carotene, or 66 or 100 mg of natural β-carotene on alternate days, see Experimental procedures) did not differ significantly. Likewise, stratification of the results according to smoking status did not yield any significant differences. Therefore, data for all study subjects were combined for the purposes of the present analyses.

Plasma β-carotene levels increased substantially among the 16 subjects during the trial period (Table 1). At baseline, subjects had an average plasma β-carotene level of 0.25 ± 0.13 μM; by day 30, the level had increased to 1.39 ± 0.54 μM. The change from baseline to day 30 was 1.14 ± 0.53 μM ($P < 0.001$). LDL β-carotene content was 0.29 ± 0.12 nmol/mg protein at baseline, and increased to 2.47 ± 1.21 nmol/mg protein at day 30, an increase of 2.18 ± 1.21 nmol/mg protein ($P < 0.001$).

Table 1
Plasma and LDL levels (mean ± S.D.) of β-carotene following supplementation in vivo for 30 days ($n = 16$)

	Baseline	Day 30	*P*
Plasma β-carotene (μM)	0.25 ± 0.13	1.39 ± 0.54	<0.001
LDL β-carotene (nmol/mg protein)	0.29 ± 0.12	2.47 ± 1.21	<0.001

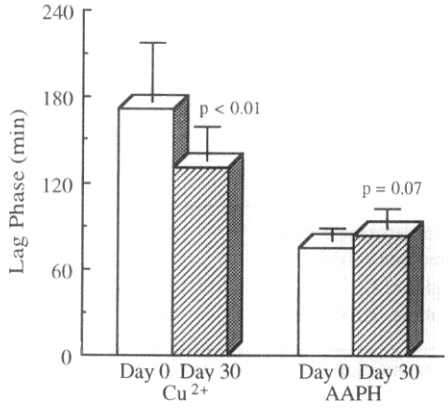

Fig. 1. Lag phase of Cu^{2+}- and AAPH-induced lipid peroxidation in LDL following β-carotene supplementation in vivo. Sixteen subjects were supplemented for 30 days with β-carotene, and LDL was isolated from plasma before and after supplementation. LDL was subjected in vitro to oxidation by Cu^{2+} or the aqueous radical-initiator AAPH, and lipid peroxidation was followed at 234 nm. The lag phase was determined as described in Experimental procedures.

Fig. 1 shows the susceptibility of LDL to oxidation as measured by the length of the lag phase preceding the propagation phase of lipid peroxidation (234 nm absorbance). For LDL exposed to the aqueous radical-initiator AAPH, there was no significant increase in the lag phase after in vivo β-carotene supplementation: the lag phase was 83 ± 14 min after supplementation, as compared to 75 ± 8 min at baseline ($P = 0.07$). For Cu^{2+}-induced lipid peroxidation, the lag phase was 172 ± 41 min at baseline, and dropped to 130 ± 24 min after supplementation ($P < 0.01$). This 25% decrease in the lag phase of Cu^{2+}-induced lipid peroxidation in LDL was significantly greater than the 7.5% coefficient of variation for the assay (see Experimental procedures). The rate of lipid peroxidation during the propagation phase in Cu^{2+}-exposed LDL before and after β-carotene supplementation was 5.66 ± 1.02 and 6.31 ± 1.56 nmol dienes/min and mg protein, respectively ($P = 0.22$).

3.2. In vitro supplementation

Similarly to in vivo supplementation, in vitro supplementation with β-carotene did not result in

Table 2
Effects of in vitro β-carotene supplementation on Cu^{2+}- and AAPH-induced oxidation of LDL

	Supplementation of LDL		Supplementation of plasma followed by LDL isolation	
	Vehicle control	Supplemented	Vehicle control	Supplemented
LDL β-carotene content (nmol/mg protein)	0.37 ± 0.08	(plus 10.0)[a]	0.56 ± 0.24	2.79 ± 0.49*
Lag phase, Cu^{2+} (min)	145 ± 37	144 ± 33	132 ± 31	134 ± 36
Lag phase, AAPH (min)	N.D.	N.D.	68 ± 11	75 ± 12[b]

Supplementation of LDL was done by adding β-carotene in hexane or tetrahydrofuran (25 μl/ml incubation) to LDL in phosphate-buffered saline, pH 7.4. Plasma supplementation was done by adding β-carotene (21.5 mM stock solution in tetrahydrofuran) to plasma (25 μl/ml plasma), followed by gel filtration, isolation of LDL, and treatment of the LDL preparation with Ebselen and GSH (see Experimental procedure). LDL was incubated with Cu^{2+} or AAPH, and the lag phase preceding the rapid propagation phase of lipid peroxidation was determined. Results represent the mean ± S.D. for three separate LDL preparations. All measurements were done in duplicate. N.D., not determined.

[a]β-Carotene was added to LDL incubations, but LDL was not re-isolated and β-carotene content determined.

*$P < 0.005$, [b]$P = 0.13$ compared with vehicle control.

increased resistance of LDL to oxidation. When a large excess (27-fold endogenous levels) of β-carotene in either hexane or THF was added to LDL in PBS, no significant change was observed in the length of the lag phase of Cu^{2+}-induced lipid peroxidation (Table 2). Likewise, when plasma was incubated with β-carotene prior to LDL isolation, resulting in LDL β-carotene levels (2.79 ± 0.49 nmol/mg protein) that were slightly higher than those achieved by in vivo supplementation (2.47 ± 1.21 nmol/mg protein, see above), the resistance of LDL to both Cu^{2+}- and AAPH-induced oxidation was not significantly increased (Table 2).

4. Discussion

In the present study we examined whether β-carotene supplementation in vivo and in vitro can increase the resistance of human LDL to metal ion-dependent and -independent oxidation. Despite substantial increases in both plasma and LDL β-carotene levels after in vivo supplementation, the resistance of LDL to either type of oxidant stress was not significantly increased. Furthermore, there was no significant protective effect of in vitro β-carotene supplementation of LDL directly, or of plasma followed by LDL isolation.

Our data on the resistance of LDL to Cu^{2+}-induced oxidation after in vivo β-carotene supplementation are in agreement with two previous studies which also failed to demonstrate a substantial protective effect [14,15]. Princen et al. [14] reported only minimally increased resistance of LDL to Cu^{2+}-induced oxidation, despite a 17-fold increase in LDL β-carotene content. Similarly, Reaven et al. [15] examined the oxidative susceptibility of LDL after supplementation with β-carotene for 3 months and found that β-carotene levels in LDL increased nearly 20-fold, but susceptibility to Cu^{2+}-induced oxidation did not decrease. On the contrary, β-carotene supplementation was associated with significantly increased ($P < 0.001$) susceptibility of LDL to Cu^{2+}-induced modification as assessed by the rate of LDL degradation by macrophages [15]. The latter result is consistent with our observation that the lag phase of Cu^{2+}-induced lipid peroxidation in LDL was significantly decreased ($P < 0.01$) following in vivo β-carotene supplementation (Fig. 1). These findings may reflect a pro-oxidant effect of increased β-carotene levels (at least at ambient oxygen pressure present during the in vitro assay of LDL oxidation, see below and [9]), or changes in lipid composition of LDL associated with β-carotene supplementation that affect LDL's susceptibility to Cu^{2+}-induced oxidation.

The study of Reaven et al. also investigated endothelial cell-mediated modification of LDL, with essentially the same results as those obtained with Cu^{2+}, i.e. no protective effect of in vivo β-carotene supplementation, but rather significantly increased ($P < 0.001$) rate of uptake of cell-modified LDL by macrophages [15]. While both Cu^{2+}-induced and endothelial cell-mediated oxidation of LDL are metal ion-dependent, we also used a metal ion-independent type of LDL oxidation, viz. aqueous peroxyl radicals generated at a constant rate by the radical initiator AAPH. It is important to investigate both types of LDL oxidation, i.e. metal ion-dependent and -independent, as the mechanism of LDL oxidation in vivo is at present unknown. However, β-carotene supplementation in vivo did not significantly increase LDL's resistance to AAPH-induced oxidation (Fig. 1).

Our data showing a lack of an effect of in vitro supplementation with β-carotene against LDL oxidation are in agreement with two previous in vitro studies [10,11], but are in disagreement with two other studies [12,13]. Jialal et al. [12] found that β-carotene supplementation of LDL in vitro strongly inhibited oxidation induced by Cu^{2+} or human monocyte-derived macrophages, and similar observations were recently reported by Lavy et al. using Cu^{2+} [13]. We were unable to confirm these results although we used similar methods of β-carotene supplementation as these authors, viz. direct addition of β-carotene in organic solvent to LDL in aqueous solution [12,13], or addition of β-carotene dissolved in THF to plasma prior to isolation of LDL [13]. Addition of β-carotene in hexane or THF to LDL in PBS may not only exert a solvent effect and change lipoprotein structure and susceptibility to oxidation, but may also not have the desired effect of incorporating β-carotene into the LDL particle. Interestingly, however, both groups presented evidence that direct addition of β-carotene to LDL resulted in tight association of the added β-carotene with the lipoprotein [13,24]. In contrast, Esterbauer et al. [25] demonstrated that adding an ethanolic solution of α-tocopherol to LDL in aqueous solution does not result in effective incorporation of this lipid-soluble compound into LDL. However, supplementation of plasma with α-tocopherol followed by isolation of LDL resulted in effective enrichment of the lipoprotein with α-tocopherol [25]. A similar method was used by us and also by Lavy et al. [13] to enrich LDL with β-carotene. Although the LDL β-carotene levels achieved by this method were similar in our study (2.79 ± 0.49 nmol/mg protein) and [13] (2.14 ± 0.18 nmol/mg protein), we, in contrast to [13], did not observe inhibition of Cu^{2+}-induced lipid peroxidation in LDL. This discrepancy may be explained by differences in the levels of pre-formed lipid hydroperoxides in our and their LDL preparations. We observed that incubation of plasma with β-carotene or the vehicle THF leads to formation of varying amounts of lipid hydroperoxides in LDL (see Experimental procedures). As it is known that the amounts of pre-formed lipid hydroperoxides is an important factor in determining the susceptibility of LDL to Cu^{2+}-induced oxidation [16,18], we removed lipid hydroperoxides by treatment with Ebselen and GSH prior to LDL incubation with Cu^{2+}. This step, which in our hands proved pivotal to obtaining reproducible results, was not taken by Lavy et al. [13]. Therefore, it is possible that in their experiments control LDL isolated from vehicle-treated plasma had substantially higher levels of pre-formed lipid hydroperoxides than LDL isolated from β-carotene-treated plasma, and consequently the control LDL exhibited a shorter lag phase than the β-carotene-enriched LDL.

Interestingly, in vivo β-carotene supplementation in our study was associated with significantly decreased resistance of LDL to Cu^{2+}-induced oxidation (see above), but no reduction in the lag phase was observed after in vitro supplementation (Table 2). This may indicate that β-carotene supplementation in vivo results in changes in LDL which increase its susceptibility to Cu^{2+}-induced oxidation that are not mimicked by addition of β-carotene in vitro. It may be that in vivo supplementation affects not only LDL β-carotene levels but also lipid metabolism and thus LDL lipid composition or structure. We did not observe a similar discrepancy between the effects of in vivo and in vitro β-carotene supplementation on AAPH-induced LDL oxidation: in both cases, LDL resistance to oxidation was slightly increased following β-carotene supplementation (see Fig. 1 and Table 2). Taken together, these data reinforce our earlier report [16] that LDL oxidation by

metal ion-dependent and -independent processes behave quite independently.

It remains possible that β-carotene contained within the LDL particle can inhibit LDL oxidation in vivo, but the in vitro assays used to measure LDL oxidation do not readily mimic the in vivo process. For example, it has been reported that β-carotene exhibits effective radical-trapping antioxidant activity at low (physiologic) oxygen tensions (e.g. 15 torr), but not at ambient or higher oxygen partial pressures (>150 torr) [9]. Therefore, assessment of LDL oxidation in vitro at ambient oxygen tension may not be the appropriate setting to test β-carotene's antioxidant effect. (However, we have found no protective effect of in vitro β-carotene supplementation of plasma followed by LDL isolation on Cu^{2+}-induced oxidation of LDL both at 150 and 15 torr (A. Hatta and B. Frei, unpublished observations)). Other important concerns have been raised recently about in vitro studies of LDL oxidation, such as rates of radical production, LDL concentration in the incubation medium, and the absence of other physiological antioxidants such as vitamin C and ubiquinol, which are all factors that substantially affect the lipid peroxidation lag phase in LDL and may obscure a protective effect of β-carotene [26]. It is also possible that β-carotene inhibits oxidation of LDL in vivo not from within the particle but by reducing oxidative stress in the atherosclerotic lesion where carotenoids accumulate [27]. Indeed, treatment of endothelial cells and smooth muscle cells in culture with β-carotene has been shown to inhibit minimal oxidative modification of LDL, in contrast to treatment of LDL with β-carotene [28]. The authors of the study suggested that this effect may be due to decreased cellular formation of reactive oxygen species, or reduced production of oxidized cellular lipids which may otherwise be transferred to LDL and contribute to its oxidation. Finally, it could be that β-carotene does not have the ability to inhibit oxidation of LDL, but may function in a different way to prevent or delay the progression of atherosclerosis. For example, it was reported recently that in cholesterol-fed rabbits β-carotene supplementation preserved endothelial function, which was related to increased arterial tissue levels of β-carotene but not to protection of LDL against oxidation [29]. There is also suggestive evidence that antioxidants may reduce platelet aggregability [30] and alter lipoprotein profile [4,31], which may be independent of their ability to protect LDL from oxidation.

In summary, the present study shows that β-carotene supplementation, either in vivo or in vitro, does not protect LDL from either metal ion-dependent or -independent oxidation. Further research will be required to determine whether or not β-carotene can protect LDL from oxidation in the arterial wall, or whether other specific or nonspecific functions of β-carotene are responsible for its effects on the development of cardiovascular disease.

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References

[1] Steinberg, D., Antioxidant vitamins and coronary heart disease, N. Engl. J. Med., 328 (1993) 1487.

[2] Manson, J.E., Stampfer, M.J., Willett, W.C. et al., A prospective study of antioxidant vitamins and incidence of coronary heart disease in women, Circulation, 84 (Suppl. II) (1991) II-546.

[3] Rimm, E.B., Stampfer, M.J., Ascherio, A., Giovannucci, E., Colditz, G.A. and Willett, W.C., Vitamin E consumption and the risk of coronary heart disease in men, N. Engl. J. Med., 328 (1993) 1450.

[4] Kardinaal, A.F.M., Kok, F.J., Ringstad, J. et al., Antioxidants in adipose tissue and risk of myocardial infarction: the EURAMIC study, Lancet, 342 (1993) 1379.

[5] Gaziano, J.M., Manson, J.E., Ridker, P.M., Buring, J.E. and Hennekens, C.H., Beta carotene therapy for chronic stable angina, Circulation, 82 (Suppl. III) (1990) III-201.

[6] The ATBC Cancer Prevention Study Group, The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers, N. Engl. J. Med., 330 (1994) 1029.

[7] Palozza, P. and Krinsky, N.I., Antioxidant effects of carotenoids in vitro and in vivo: an overview, Methods Enzymol., 213 (1992) 403.

[8] Foote, C.S. and Denny, R.W., Chemistry of singlet oxygen VIII. Quenching by β-carotene, J. Am. Chem. Soc., 90 (1968) 6233.

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

[10] Morel, D.W., Hessler, J.R. and Chisolm, G.M., Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid, J. Lipid Res., 24 (1983) 1070.

[11] van Hinsbergh, V.W.M., Scheffer, M., Havekes, L. and Kempen, H.J.M., Role of endothelial cells and their products in the modification of low-density lipoproteins, Biochim. Biophys. Acta, 878 (1986) 49.

[12] Jialal, I., Norkus, E.P., Cristol, L. and Grundy, S.M., β-Carotene inhibits the oxidative modification of low-density lipoprotein, Biochim. Biophys. Acta, 1086 (1991) 134.

[13] Lavy, A., Ben Amotz, A. and Aviram, M., Preferential inhibition of LDL oxidation by the all-trans isomer of β-carotene in comparison with 9-cis β-carotene, Eur. J. Clin. Chem. Clin. Biochem., 31 (1993) 83.

[14] Princen, H.M.G., van Poppel, G., Vogelezang, C., Buytenhek, R. and Kok, F.J., Supplementation with vitamin E but not β-carotene in vivo protects low density lipoprotein from lipid peroxidation in vitro. Effect of cigarette smoking, Arteriosclerosis Thromb., 12 (1992) 554.

[15] Reaven, P.D., Khouw, A., Beltz, W.F., Parthasarathy, S. and Witztum, J.L., Effect of dietary antioxidant combinations in humans. Protection of LDL by vitamin E but not by β-carotene, Arteriosclerosis Thromb., 13 (1993) 590.

[16] Frei, B. and Gaziano, J.M., Content of antioxidants, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependent and -independent oxidation, J. Lipid Res., 34 (1993) 2135.

[17] Frei, B., Yamamoto, Y., Niclas, D. and Ames, B.N., Evaluation of an isoluminol chemiluminescence assay for the detection of hydroperoxides in human blood plasma, Anal. Biochem., 175 (1988) 120.

[18] O'Leary, V.J., Darley-Usmar, V.M., Russell, L.J. and Stone, D., Pro-oxidant effects of lipoxygenase-derived peroxides on the copper-initiated oxidation of low-density lipoprotein, Biochem. J., 282 (1992) 631.

[19] Lynch, S.M. and Frei, B., Mechanisms of copper- and iron-dependent oxidative modification of human low density lipoprotein, J. Lipid Res., 34 (1993) 1745.

[20] Stocker, R., Bowry, V.W. and Frei, B., Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does α-tocopherol, Proc. Natl. Acad. Sci. USA, 88 (1991) 1646.

[21] Johnson, E.J. and Russell, R.M., Distribution of orally administered β-carotene among lipoproteins in humans, Am. J. Clin. Nutr., 56 (1992) 128.

[22] Davies, B.H., Carotenoids. In: Goodwin, T.W. (Ed.), Chemistry and Biochemistry of Plant Pigments, Vol. 2, Academic Press, London, 2nd Edn., 1976, pp. 38–165.

[23] Esterbauer, H., Gebicki, J., Puhl, H. and Jürgens, G., The role of lipid peroxidation and antioxidants in oxidative modification of LDL, Free Radical Biol. Med., 13 (1992) 341.

[24] Jialal, I. and Grundy, S.M., Influence of antioxidant vitamins on LDL oxidation, Ann. NY Acad. Sci., 669 (1992) 237.

[25] Esterbauer, H., Dieber-Rotheneder, M., Striegl, G. and Waeg, G., Role of vitamin E in preventing the oxidation of low-density lipoprotein, Am. J. Clin. Nutr., 53 (1991) 314S.

[26] Ingold, K.U., Bowry, V.W., Stocker, R. and Walling, C., Autoxidation of lipids and antioxidation by α-tocopherol and ubiquinol in homogeneous solution and in aqueous dispersions of lipids: unrecognized consequences of lipid particle size as exemplified by oxidation of human low density lipoprotein, Proc. Natl. Acad. Sci. USA, 90 (1993) 45.

[27] Prince, M.R., LaMuraglia, G.M. and MacNichol, E.F.J., Increased preferential absorption in human atherosclerotic plaque with oral beta carotene. Implications for laser endarterectomy, Circulation, 78 (1988) 338.

[28] Navab, M., Imes, S.S., Hama, S.Y. et al., Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein 1 synthesis and is abolished by high density lipoprotein, J. Clin. Invest., 88 (1991) 2039.

[29] Keaney, J.F., Jr. , Gaziano, J.M., Xu, A. et al., Dietary antioxidants preserve endothelium-dependent vessel relaxation in cholesterol-fed rabbits, Proc. Natl. Acad. Sci. USA, 90 (1993) 11 880.

[30] Oda, T., Ikeda, H., Kuwano, K. and Nakayama, H., Alpha-tocopherol inhibits platelet and neutrophil activation during cyclic flow variations in dogs with coronary stenosis, Circulation, 88 (Suppl. I) (1993) I-35.

[31] Gaffney, P.T., Buttenshaw, R.L., Lovell, G.A., Kerswill, W.J. and Ward, M., β-Carotene supplementation raises serum HDL-cholesterol, Aust. N.Z. J. Med., 20 (Suppl. I) (1990) I-365.