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Original Contribution

ENHANCED RESISTANCE TO OXIDATION OF LOW DENSITY LIPOPROTEINS AND DECREASED LIPID PEROXIDE FORMATION DURING β-CAROTENE SUPPLEMENTATION IN CYSTIC FIBROSIS

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Abstract We investigated the effect of correcting β-carotene deficiency in cystic fibrosis (CF) patients on two parameters of lipid peroxidation: The resistance to oxidation of low density lipoprotein (LDL) was measured by the lag time preceding the onset of conjugated diene formation during exposure to copper(I) ions, and lipid peroxide formation was quantitated by malondialdehyde concentrations in plasma (TBA/IDPLC method). Simultaneously, α-tocopherol and β-carotene concentrations were determined in LDL and in plasma. Thirty-four CF patients were investigated before and after 3 months of oral β-carotene supplementation. β-carotene concentrations increased (p < 0.0001) in plasma (mean ± SD) (0.09 ± 0.06 vs. 1.07 ± 0.86 μmol/l) and in LDL (0.02 ± 0.02 vs. 0.31 ± 0.28 μmol/mol), without significant changes in α-tocopherol, either in plasma (24.7 ± 5.9 vs. 25.4 ± 7.0 μmol/l) or in LDL (4.47 ± 2.95 vs. 4.05 ± 4.13 μmol/mol). Lag times, being shorter (p < 0.05) in patients than in controls, increased from 48.5 ± 21.3 to 69.1 ± 27.9 min (p < 0.001) and plasma MDA concentrations, being greater (p < 0.0001) in patients than in controls, decreased from 0.95 ± 0.32 to 0.61 ± 0.15 μmol/l (p < 0.0001). At 3 months, lag times and MDA concentrations did not any longer differ between patients and controls. These data suggest that excess lipid peroxidation occurring in β-carotene deficiency can be limited and normalized during efficient β-carotene supplementation in CF patients.

Keywords α-Tocopherol, β-Carotene, Lipid peroxidation, Conjugated dienes, LDL, Malondialdehyde, Free radicals, Cystic fibrosis

INTRODUCTION

A growing number of studies addressed the effect of β-carotene on the resistance to oxidation of low density lipoprotein (LDL) in subjects with a normal or marginal β-carotene status prior to supplementation, resulting in extremely high β-carotene values up to 10 to 20 times normal, which hardly reflect a physiological but rather an experimental condition. In contrast, only limited data are available allowing us to answer the question of whether an almost complete lack of β-carotene in human subjects substantially deteriorates the antioxidant protection of polyunsaturated fatty acids in LDL and biomembranes and thus leads to increased lipid peroxide formation. The same holds true for the effects of correcting this deficiency, mainly due to the fact that severe β-carotene deficiency is limited to a few pathological conditions, comprising an impaired intestinal absorption of this micronutrient, or to healthy subjects after experimental depletion.

Cystic fibrosis (CF) patients frequently exhibit severe β-carotene deficiency due to exocrine pancreatic insufficiency and thus represent a clinical setting for investigating the role of β-carotene on lipid peroxidation. In view of increased free radical generation from inflammatory cells in chronic lung inflammation on one hand and combined antioxidant deficiencies on the other, CF patients are at increased risk of exhibiting a shift in the oxidant–antioxidant balance in favor of the former. We hypothesized that β-carotene deficiency aggravates this imbalance, leading to excess lipid peroxidation, which in turn could result in enhanced propagation of disease processes, for example, in the lung and in the liver of these patients.

We first addressed the question of whether LDL of
vitamin E-sufficient but β-carotene-deficient CF patients exhibits an impaired resistance to oxidation and if, after correction of β-carotene deficiency in vivo, isolated LDL, the main carrier of β-carotene in the blood, can withstand this oxidative stress more efficiently than before supplementation. Second, we focused on the hypothesis that, due to the oxidant-antioxidant imbalance in favor of the former, plasma lipid peroxide levels are increased in CF patients with uncorrected β-carotene deficiency, but can be decreased during supplementation. Thus, this study was designed to answer the question of whether β-carotene supplementation of vitamin E-sufficient but β-carotene-deficient CF patients would result in an enhanced protection against lipid peroxidation. If so, this could provide the basis for a new therapeutic concept for patients with this complex inherited metabolic disorder. In addition, the results obtained in this study are expected to contribute to the present knowledge about the biological properties of β-carotene.

METHODS

Study design

The oxidation resistance of and the antioxidant concentrations in LDL were determined in β-carotene-deficient CF patients at study entry (baseline values) and after 3 months of β-carotene supplementation. Plasma concentrations of MDA, antioxidants, and cholesterol were quantitated at study entry, after 3 weeks and 3 months of treatment. Controls had a single evaluation, including the same parameters as assessed in patients, to allow, by comparison, validation of baseline values of patients and evaluation of treatment efficacy.

Subjects

Patients. CF patients, under long-term care in the CF outpatients clinic of the Department of Pediatrics, University of Zurich, with plasma β-carotene concentrations < 0.32 μmol/l (mean − 1 SD of age-matched Swiss controls, Winklhofer-Roob et al., unpublished data) and enrolled in a β-carotene supplementation trial, were eligible for inclusion in this investigation if they had both LDL and plasma α-tocopherol concentrations above mean − 2 SD of healthy subjects, that is, for LDL, 2.22 mol/mol, derived from mean = 7.26, SD = 2.52 mol/mol, established for a healthy Austrian population in the same laboratory where the present investigations were done and for plasma, 15.9 μmol/l, derived from mean = 29.3, SD = 6.7 μmol/l, established by Vuilleumier et al. in the laboratories of the Vitamin Research Department of Hoffmann-La Roche Ltd., Basel, Switzerland, where the plasma antioxidant measurements in this study were performed. Thirty-four of 37 patients enrolled in the β-carotene supplementation study, 18 M, 16 F, ages 10.8 ± 7.6 years, fulfilled the inclusion criteria. All had been on oral vitamin E supplements (330 ± 120 IU daily, RRR-α-tocopherol, MULTABEN® Roche Pharma Ltd., Basel, Switzerland, or all-rac-α-tocopheryl acetate, EPHYNAL®, Hoffmann-La Roche Ltd., Basel, Switzerland) at least during one year prior to this study. The diagnosis of CF had been established by typical clinical symptoms and elevated sweat electrolytes. The patients’ Shwachman scores were 84 ± 12. All patients were in a stable disease status, none had an acute exacerbation at any of the three evaluations. All patients exhibited exocrine pancreatic insufficiency, which was treated with pancreatic enzymes (CREON®, KaliChemie Ltd., Bern, Switzerland, or PANZYTAT 25000®, Knoll Ltd., Liestal, Switzerland), the dosages (310,000 ± 260,000 FIP units) being individually adjusted to the results of repeated fat balance studies prior to study entry. The actual coefficients of fat absorption were 83.0 ± 10.3%. Patients were on a free diet containing 30–40% of energy as fat as evaluated from repeated 72 h-food records before enrollment in this investigation. At study entry, 17 patients were taking 200–2000 ml (890 ± 550 kcal/day) of liquid formulas (FRESUBIN®, Fresenius Ltd., Stans, Switzerland, NUTRODRI®, Sandoz-Wander Ltd., Bern, Switzerland, or TWOCAL®, Abbott Ltd., Cham, Switzerland) as part of the therapeutic regime routinely applied in order to meet the caloric needs and did so throughout the study period. No other changes were introduced into the diet. Three patients who were on long-term oral retinyl palmitate supplements (AROVIT®, Hoffmann-La Roche Ltd.) and nine patients who were on a multivitamin preparation (PROTOVIT®, Hoffmann-La Roche Ltd.) continued to take the vitamins. None had had β-carotene supplements prior to study entry. Other medications, included in the overall therapeutic regime, for example, antibiotics, applied orally and/or by aerosol to 10/32 patients at study entry and to 4 additional patients after 3 months, were maintained without introduction of new drugs during the study period. Clinical characteristics of patients at baseline and after 3 months of supplementation, comprising nutritional (weight, upper arm circumference; albumin, prealbumin) and infectious parameters (C-reactive protein, α1-acid glycoprotein, bands as % of WBC), did not change. Two patients were lost to follow-up. For five other patients, either baseline (n = 4) or 3 month values (n = 1) for the LDL resistance to oxidation were not available. Thus, data from 27 and 32 patients for determinations of the LDL resistance to oxidation and plasma MDA concentrations, respectively, could be included in statistical analysis.
Carotene and lipid peroxidation in CT

Controls. Forty-two clinically healthy staff members of the Department of Pediatrics, University of Zurich, 15 M, 27 F, ages 31.5 ± 8.0 years, were recruited to participate in this study, as it was ethically not acceptable to draw the necessary amounts of blood from healthy children. All persons were on a usual mixed diet, all but two were non-smokers, all but one (who took regularly 500 mg vitamin C daily) did not use vitamin supplements. Twelve women were on oral contraceptives. Twenty-two of these volunteers, 10 M, 12 F, ages 35.1 ± 8.2 years, had also determinations of the LDL resistance to oxidation; for two of them, data on the LDL content of antioxidants were missing. From one volunteer no blood was available for the measurement of plasma MDA concentrations. In the plasma sample obtained from another volunteer, hemolysis had occurred. As this has been reported to influence the MDA-TBA test results, this person was excluded from further evaluation. Consequently, data from 40 subjects for MDA concentrations were included in statistical analysis.

Therapeutic intervention

During the study period, patients received β-carotene orally in a daily dosage of 0.5 mg/kg body weight, given during breakfast in capsules containing 5 mg synthetic β-carotene as 10% water-miscible beadlets, with 80% all-trans and 20% cis isomers (BEILACAROTIN®, 3M Medica, Ltd., Borken, Germany).

Ethical considerations

The study was approved by the Ethical Committee of the Department of Pediatrics, University of Zurich, and informed consent was obtained from the patients or their parents as well as from healthy controls.

Plasma sampling and preparation of LDL

After an overnight fast, blood was drawn in sterile plastic tubes, containing K-EDTA (1.6 mg/ml) as an anticoagulant (Sarstedt Monovette®, Nümbrecht, Germany), protected from light by aluminum foil and centrifuged immediately at 2000 g for 8 min. Plasma was separated and samples were divided into aliquots for the different determinations. While kept cool on ice, an aliquot of EDTA supplemented plasma from each patient was shipped by overnight courier, and within 36 h after blood was drawn, LDL was isolated by density gradient ultracentrifugation in a density range of 1.02 - 1.05 g/ml (swingout rotor SW-41, 40000 rpm, 10–15°C, 20–24 h). LDL was then removed and divided into aliquots for quantification of antioxidants and determination of the LDL resistance to oxidation.

Determination of the LDL resistance to oxidation

Immediately after isolation, LDL was processed for determination of the resistance to oxidation. Separation of LDL from EDTA and salts of the density gradient was performed by gel-filtration using Biogel P6-DG (EconoPac DG columns, BioRad, Inc., Hercules, CA). LDL was diluted to 0.25 mg/ml LDL mass (corresponding to 50 µg LDL protein) in oxygen saturated phosphate buffer saline, pH 7.4. Oxidation was initiated by exposure of LDL to 16.6 μM Cu²⁺, and CD absorbance at 234 nm was recorded spectrophotometrically (UV 1202, Shimadzu, Inc., Japan) in 1 cm cuvettes with automatic cuvette changing in 2 min intervals. The relative increase in CD absorbance was continuously monitored until LDL was fully oxidized. From the 234 nm trace, the lag time (Index I of the oxidation resistance of LDL) was determined as the intercept of the tangent on the first turning point with the time axis. The maximum rate of oxidation (Index II of the oxidation resistance), corresponding to the slope at the turning point, was calculated (ΔA/min), and the time of occurrence was recorded. Finally, the maximum increase in CD absorbance (Index III) and the corresponding time point were determined. This assay has proved to be a reliable and reproducible method for the determination of the oxidation resistance of LDL, with an intra-assay coefficient of variation of 1.9% and an inter-assay coefficient of variation of 4.4%.

Determination of antioxidants in LDL

Aliquots of isolated LDL samples containing EDTA were stored at −70°C until α- and γ-tocopherol and carotenoids (β-carotene, lycopene, lutein/zeaxanthin, cryptoxanthin) were determined by different HPLC methods: for tocopherols as described by Vuilleumier et al. and Esterbauer et al. and for carotenoids by a modified method of Bieri et al. Acetonitrile: methylenechloride: methanol = 67:19:14 was used as mobile phase with a flow rate of 1.3 ml/min. All concentrations were expressed as mol/mol total LDL.

Determination of antioxidants in plasma

Another portion of plasma was kept frozen at −20°C for a maximum of 4 days before determination of plasma antioxidants (α-tocopherol, α- and β-carotene, lycopene) by HPLC according to Hess et al.
Table 1. Antioxidants in LDL and Parameters of LDL Oxidation in Patients at Baseline and After 3 Months of Supplementation as Well as in Healthy Controls

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 27)</th>
<th>Controls (n = 22)</th>
<th>p Values</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Baseline A</td>
<td>3 Months B</td>
<td>C</td>
</tr>
<tr>
<td>I. Antioxidants in LDL (mol/mol)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>(\alpha)-Tocopherol</td>
<td>8.47 ± 2.95</td>
<td>9.05 ± 4.13</td>
<td>6.40 ± 1.26</td>
</tr>
<tr>
<td>(\beta)-Carotene</td>
<td>0.02 ± 0.02</td>
<td>0.31 ± 0.28</td>
<td>0.31 ± 0.24</td>
</tr>
<tr>
<td>II. Parameters of LDL oxidation</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Lag time (min)</td>
<td>48.5 ± 21.3</td>
<td>69.1 ± 27.9</td>
<td>62.5 ± 22.8</td>
</tr>
<tr>
<td>Maximum rate of oxidation dA/min</td>
<td>0.026 ± 0.007</td>
<td>0.026 ± 0.008</td>
<td>0.023 ± 0.006</td>
</tr>
<tr>
<td>At time (min)</td>
<td>69.3 ± 23.9</td>
<td>89.6 ± 33.3</td>
<td>84.3 ± 26.7</td>
</tr>
<tr>
<td>Maximum increase in CD absorbance A234 nm</td>
<td>0.83 ± 0.11</td>
<td>0.82 ± 0.14</td>
<td>0.77 ± 0.08</td>
</tr>
<tr>
<td>At time (min)</td>
<td>98.8 ± 26.8</td>
<td>119.1 ± 38.3</td>
<td>117.4 ± 34.2</td>
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</table>

\(dA/\text{min} = \text{Changes in conjugated diene absorbance/min; CD = Conjugated dienes; NS = not significant. Values are expressed as mean ± SD.}\)
\(^a\) Data on antioxidants in LDL were obtained in 20 controls.
\(^b\) Wilcoxon matched pairs signed ranks tests, two-tailed.
\(^c\) Mann–Whitney U tests, two-tailed.

**Determination of lipid peroxides in plasma**

Another aliquot of EDTA plasma was stored at \(-70^\circ\text{C}\) until lipid peroxides were determined by HPLC measurement of the MDA-TBA adduct according to Wong et al.\(^{21}\) as described previously.\(^{29}\) Coefficients of variations of repeated MDA-TBA assays in our laboratory were 4.4% (within run) and 6.9% (from run to run), respectively (unpublished data).

**Determination of plasma cholesterol concentrations**

Plasma cholesterol concentrations were measured enzymatically, using commercially available kits from Beckman, Ltd., Brea, CA, for the patient group and from Merck, Ltd., Darmstadt, Germany, for the control group. Ratios of plasma \(\alpha\)-tocopherol to cholesterol were calculated to correct for differences in cholesterol concentrations between patients and controls. HDL cholesterol was determined with the CHOD-PAP kit from Boehringer Mannheim, Ltd., Mannheim, Germany, and LDL and VLDL cholesterol with a kit from BioMerieux Suisse SA, Geneva, Switzerland.

**Statistical analysis**

Statistical analysis was performed at the Medical Statistics Department, University of Nijmegen. Due to nonconformity of the data with the normality assumption, Wilcoxon matched pairs signed ranks tests were used to compare study variables in patients at baseline with those after 3 months of supplementation, Mann–Whitney U tests for the corresponding comparisons between patients and controls, and Spearman rank correlation coefficients were calculated for the associations between different variables. Log-transformation was performed to obtain normality for further analysis. Multiple regression analysis was used to analyze the relationship between parameters of lipid peroxidation (lag time and plasma MDA values) and antioxidants. To further estimate the efficiency of \(\beta\)-carotene, correction for changes in LDL \(\alpha\)-tocopherol was made on the basis of the results of regression analysis. Statgraphics (STSC, Inc., Rockville, MD) Version 6 was used for all statistical procedures. Differences were considered significant at \(p < 0.05\). All results are expressed as mean ± SD unless otherwise stated.

**RESULTS**

**Antioxidants in LDL**

\(\alpha\)- and \(\gamma\)-Tocopherol. At study entry, LDL \(\alpha\)-tocopherol concentrations of patients were significantly higher than those of controls due to long-term vitamin E supplementation prior to this study. For the whole study group, they did not change significantly between baseline and 3 months (Table 1, Fig. 1A). In individual patients, however, changes in \(\alpha\)-tocopherol greater than 10% of baseline values did occur during \(\beta\)-carotene supplementation, comprising both increases (\(n = 11, 35 ± 7\%\)) and decreases (\(n = 6, 40 ± 28\%\)). Levels of \(\gamma\)-tocopherol were lower \((p < 0.0001)\) in patients \((0.15 ± 0.17 \text{ mol/mol})\) than in controls \((0.31 ± 0.17 \text{ mol/mol})\) at study entry and remained low throughout the study period.
**β-carotene and lipid peroxidation in CF**

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**β-Carotene.** Baseline LDL β-carotene concentrations of patients were significantly lower ($p < 0.0001$) than those of controls (Table 1, Fig. 1B), with values close to the detection limit in a considerable proportion of patients. They were also far below published mean values (0.29 mol/mol) of healthy subjects. After 3 months of β-carotene supplementation, LDL β-carotene concentrations of patients had significantly increased ($p < 0.0001$) by 0.29 ± 0.28 mol/mol LDL and did not differ any longer from those of controls.

As evidenced by the large SD, patients responded to supplementation with substantial between-subject variation. In a single patient, identified as far outlier in Figure 1B, an increase in LDL β-carotene concentration of 1.45 mol/mol was observed.

**Other carotenoids.** At study entry, patients showed significantly lower ($p < 0.0001$) LDL contents of lycopene (0.04 ± 0.04 mol/mol), lutein/zeaxanthin (0.002 ± 0.002 mol/mol) and cryptoxanthin 0.005 ± 0.001 mol/mol) than controls (0.37 ± 0.16, 0.012 ± 0.004, and 0.076 ± 0.049 mol/mol, respectively). In patients, lycopene and lutein/zeaxanthin concentrations did not change in LDL between study entry and 3 months, whereas cryptoxanthin levels increased about twofold ($p < 0.0001$) to 0.010 ± 0.007 mol/mol, but still remained very low compared to those of healthy subjects.

**LDL Resistance to oxidation**

Parameters of LDL oxidation, as characterized by the CD absorbance curve, that is, lag time (Index I of the oxidation resistance), maximum rate of oxidation (Index II), and maximum increase in CD absorbance (Index III), are presented for the whole study group in Table 1.

At baseline, lag times were significantly ($p < 0.05$) shorter in patients than in healthy controls (Table 1). Multiple regression analysis showed that lag times were significantly related to LDL α-tocopherol concen-
trations ($p = 0.003$), but not to values of $\beta$-carotene, which was present in very small concentrations as shown in Table 1.

After 3 months of $\beta$-carotene supplementation with an increase in LDL $\beta$-carotene concentrations as described above, lag times of patients had significantly increased ($p < 0.001$) by $20.6 \pm 25.2$ min, and the resulting lag times of $69.1 \pm 27.9$ min did not any longer differ from those of controls (Table 1, Fig. 1C). The increase in lag times varied substantially between patients, as indicated by the large SD. Multiple regression analysis was unable to identify a correlation between the increase in lag times and the increase in the log of LDL $\alpha$-tocopherol concentrations ($p = 0.58$). Changes in lag times were statistically related ($p = 0.005$) to changes in LDL $\alpha$-tocopherol levels, occurring during $\beta$-carotene supplementation and comprising both increases and decreases. The resulting regression line of changes in lag times on changes in LDL $\alpha$-tocopherol concentrations showed a slope of $(mean \pm SE) 5.6 \pm 1.8$ min/mol $\alpha$-tocopherol/mol LDL, representing an index of the efficiency of $\alpha$-tocopherol. The change in lag time when the $\alpha$-tocopherol level is kept constant is indicated by the intercept of $(mean \pm SD)$ $17.4 \pm 4.3$ min (Fig. 2). Thus, changes in $\alpha$-tocopherol concentrations explained only 3.2 min out of the total increase in lag times of 20.6 min observed during the study period. Changes in lag times after correction for changes in $\alpha$-tocopherol, that is, a mean increase of $17.4$ min, were still significantly positive ($p < 0.001$). Because no other variables were changed, these changes are attributable to the changes in LDL $\beta$-carotene, the therapeutic intervention during the study period. Patients exhibiting an increase in LDL $\alpha$-tocopherol greater than 10% of baseline values, in addition to the increase in LDL $\beta$-carotene (Fig. 2, Group C, n = 11), showed greater increases in lag times than did those with changes within 10% (Group B, n = 10) or with a decrease exceeding 10% of baseline values (Group A, n = 6). In Figure 3A, line plots of changes in lag times (corrected for changes in LDL $\alpha$-tocopherol) against changes in LDL $\beta$-carotene concentrations are presented for the individual study patients. In 22 of 27 patients lag times increased during $\beta$-carotene supplementation. However, there was a great variability in the slopes, indicating a substantial between-subject variability in the efficiency of $\beta$-carotene on the increase in lag times. As a consequence, changes in LDL $\beta$-carotene concentrations per se were not able to predict the changes in lag times in the patient population investigated in this study. In view of the great between-subject variability, large scale clinical trials are needed to establish the relationship between these variables.

Neither the maximum rate of oxidation (Index II) nor the maximum increase in CD absorbance (Index III) did change during $\beta$-carotene supplementation (Table 1). As a result of a significant increase in lag times, the maximum rate of oxidation occurred later at 3 months than at study entry ($p < 0.001$), as did the maximum increase in CD absorbance ($p < 0.01$). No differences in these indices of the LDL resistance to oxidation were found between patients and controls either at study entry or after 3 months of supplementation.

Antioxidants in plasma

$\alpha$-Tocopherol. Baseline plasma $\alpha$-tocopherol concentrations were significantly lower in patients than in healthy controls (Table 2, Fig. 1D), in contrast to higher LDL $\alpha$-tocopherol values and higher ratios of plasma $\alpha$-tocopherol to cholesterol due to significantly lower cholesterol concentrations of the study patients (Table 2). Plasma $\alpha$-tocopherol levels of control subjects ($28.3 \pm 6.2$ $\mu$mol/l) were comparable to those previously reported by Vuilleumier et al. for healthy Swiss adults ($29.3 \pm 6.7$ $\mu$mol/l). During 3 months of $\beta$-carotene supplementation plasma $\alpha$-tocopherol concentrations did not change in the whole study group (Table 2). Changes greater than 10% of baseline values.
occurred in nine patients exhibiting an increase of 35 \pm 13\% and in seven patients exhibiting a decrease of 28 \pm 12\%.

**\(\beta\)-Carotene.** At study entry, patients showed significantly lower plasma \(\beta\)-carotene concentrations than study controls (Table 2, Fig. 1E). Plasma \(\beta\)-carotene levels increased rapidly up to 3 weeks, and a further small but still significant increase \((p < 0.05)\) was observed between 3 weeks and 3 months. Values achieved both at 3 weeks and 3 months did no longer differ from those of controls. The patient with an exceptionally high increase in LDL \(\beta\)-carotene exhibited a corresponding increase in plasma \(\beta\)-carotene (Fig. 1B and E). The all-trans isomer accounted almost exclusively for the increase in total plasma \(\beta\)-carotene concentrations, and only small changes in cis isomers did occur. Control subjects not taking supplements showed a similar distribution of the two isomers.

Other carotenoids. Plasma \(\alpha\)-carotene concentrations were significantly lower \((p < 0.001)\) in patients than in controls \((0.01 \pm 0.01 \mu\text{mol/l} vs. 0.27 \pm 0.19 \mu\text{mol/l})\). They increased twofold between study entry and 3 weeks and threefold between study entry and 3 months, yet the amounts detected in the patients' samples still remained extremely low \((0.02 \pm 0.02 \mu\text{mol/l} and 0.03 \pm 0.02 \mu\text{mol/l}, respectively)\). Plasma lycopene levels were significantly lower in patients than in healthy subjects at study entry \((0.07 \pm 0.08 \mu\text{mol/l} vs. 0.59 \pm 0.21 \mu\text{mol/l}, respectively)\) and throughout the observation period (3 weeks, 0.06 \pm 0.07 \mu\text{mol/l}, 3 months, 0.06 \pm 0.07 \mu\text{mol/l}).

**Plasma MDA concentrations**

Baseline plasma MDA concentrations of patients were significantly greater than those of controls (Table 2, Fig. 1F), with a maximum of 1.94 \(\mu\text{mol/l}\). Multiple regression analysis showed that the log of MDA concentrations was related neither to the log of plasma \(\beta\)-carotene concentrations \((r = 0.84)\) nor to the log of plasma \(\alpha\)-tocopherol values \((p = 0.24)\).

During \(\beta\)-carotene supplementation, plasma MDA concentrations decreased successively. MDA values at 3 weeks were already smaller than baseline values and there was a further decrease \((p < 0.01)\) between 3 weeks and 3 months. A substantial between-subject variation was observed, but overall there was a 36\% reduction of MDA values. At 3 months, MDA concentrations of patients did not any longer differ from those of controls \((0.61 \pm 0.22 \mu\text{mol/l})\) (Table 2). The latter were in good agreement with those published for healthy subjects by Wong et al. \((0.60 \pm 0.13 \mu\text{mol/l})\). Multiple regression analysis showed that, like the situation at study entry, also changes in the log of plasma MDA concentrations were neither related to changes in the log of plasma \(\beta\)-carotene values \((r = 0.97)\) nor to those in the log of plasma \(\alpha\)-tocopherol levels \((r = 0.14)\). As shown in Figure 3B, in all but three patients the increase in plasma \(\beta\)-carotene concentrations was accompanied by a decrease in plasma MDA values, but the efficiency of \(\beta\)-carotene on plasma MDA concentrations was highly subject-specific. The mean \((\pm\text{SE})\) value of the slopes calculated for the regression of plasma MDA values on plasma \(\beta\)-carotene values (measured at baseline, after 3 weeks and 3 months) for the individual patients was \(-0.40 \pm 0.07 \mu\text{mol/l} \beta\)-carotene, indicating the average effi-
Table 2. Plasma Concentrations of Antioxidants and MDA in Patients at Baseline and After 3 Months of Supplementation as Well as in Healthy Controls

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 32)</th>
<th>Controls (n = 40)</th>
<th>p Values</th>
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<tbody>
<tr>
<td></td>
<td>Baseline A</td>
<td>3 Weeks B</td>
<td>3 Months C</td>
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<tr>
<td></td>
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<td></td>
<td>D</td>
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<tr>
<td>I. Plasma antioxidants (μmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>24.7 ± 5.9</td>
<td>24.6 ± 6.6</td>
<td>25.4 ± 7.6</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.09 ± 0.06</td>
<td>0.92 ± 0.65</td>
<td>1.07 ± 0.86</td>
</tr>
<tr>
<td>cis isomers</td>
<td>&lt; DL</td>
<td>0.04 ± 0.03</td>
<td>0.05 ± 0.05</td>
</tr>
<tr>
<td>All-trans isomers</td>
<td>0.09 ± 0.06</td>
<td>0.88 ± 0.62</td>
<td>1.01 ± 0.82</td>
</tr>
<tr>
<td>II. Plasma MDA (μmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA</td>
<td>0.95 ± 0.32</td>
<td>0.72 ± 0.22</td>
<td>0.61 ± 0.15</td>
</tr>
<tr>
<td>III. Plasma cholesterol (mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>3.39 ± 0.69</td>
<td>3.25 ± 0.78</td>
<td>3.26 ± 0.85</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>1.70 ± 0.43</td>
<td>1.70 ± 0.52</td>
<td>1.72 ± 0.60</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.20 ± 0.33</td>
<td>1.22 ± 0.40</td>
<td>1.18 ± 0.37</td>
</tr>
<tr>
<td>α-Tocopherol: cholesterol</td>
<td>7.39 ± 1.73</td>
<td>7.68 ± 1.53</td>
<td>8.01 ± 2.20</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD; MDA = Malondialdehyde.

a Wilcoxon matched pairs signed ranks tests, two-tailed.

b Mann–Whitney U tests, two-tailed.

Relations of antioxidants and parameters of lipid peroxidation between LDL and plasma

Both at baseline (A) and after 3 months of β-carotene supplementation (B), α-tocopherol (1), β-carotene (2), and lycopene (3) levels in LDL were strongly correlated with plasma values (Spearman rank correlation, A: (1) \( r = 0.79, p < 0.001 \); (2) \( r = 0.76, p < 0.001 \); (3) \( r = 0.84, p < 0.001 \) and B: (1) \( r = 0.64, p = 0.004 \); (2) \( r = 0.81, p < 0.001 \); (3) \( r = 0.52, p = 0.02 \), respectively). Changes in LDL β-carotene concentrations during supplementation were positively correlated with changes in plasma β-carotene levels (\( r = 0.82, p < 0.0001 \)). In contrast, there was no correlation between changes in α-tocopherol concentrations in LDL and in plasma (\( r = -0.01, p = 0.97 \)), the changes in plasma being smaller than those in LDL (plasma, −49% to +51% vs. LDL, −84% to +350%). Changes in LDL α-tocopherol concentrations were not significantly related to changes in LDL and HDL cholesterol fractions (\( r = -0.34, p = 0.09 \)). There was also no correlation between the increase in lag times and the decrease in MDA concentrations (\( r = -0.07, p = 0.71 \)) (\( r = \) Spearman rank correlation coefficients).

DISCUSSION

This study investigates two different parameters of lipid peroxidation in two different compartments (LDL and plasma) before and after correction of severe long-standing β-carotene deficiency in vitamin E-sufficient CF patients. To our knowledge, the data presented are the first addressing changes in LDL resistance to oxidation in subjects severely depleted of β-carotene with a subsequent repletion to LDL β-carotene values of healthy individuals.

Inclusion criteria allowed only participation of patients with a normalized vitamin E status after long-term vitamin E supplementation. In the presence of low plasma cholesterol values, a frequent finding in these patients, \( \alpha \)-tocopherol to cholesterol and LDL \( \alpha \)-tocopherol values were even about 1.3 times higher in patients than in healthy subjects. Patients continued to take vitamin E supplements during the study period, and a small but statistically not significant increase in LDL \( \alpha \)-tocopherol values did occur. At study entry, lag times were significantly correlated with LDL \( \alpha \)-tocopherol values, as expected from the well-documented antioxidant action of \( \alpha \)-tocopherol in LDL. Changes in LDL \( \alpha \)-tocopherol levels in individual patients occurring during 3 months of β-carotene supplementation and comprising both increases and decreases, were also significantly correlated with changes in lag times. However, these changes explained only 3.2 min of 20.6 min overall increase in lag times achieved during the study period. Changes in LDL \( \alpha \)-tocopherol could be due to changes in compliance, an increase could also result from a protective action of β-carotene on \( \alpha \)-tocopherol, delaying the radical-initiated consumption of \( \alpha \)-tocopherol.

The data presented provide evidence for an additive
The protective effect of α-tocopherol and β-carotene as indicated by the greatest increase in lag times in patients with an increase in both antioxidants. In in vitro systems, Palozza and Kriósky demonstrated that lipid peroxidation can be delayed not only by α-tocopherol but also by β-carotene with an additive or even synergistic action of these antioxidants and discussed the concept that α-tocopherol could eventually prevent β-carotene from exerting a pro-oxidant effect, for example, through limiting the production of the β-carotene peroxyl radical, which is hypothesized to be generated during the antioxidant action of β-carotene. If this indeed plays an important role, the excellent vitamin E status of the study patients could have been contributive or even essential for the antioxidant activity of β-carotene observed in this study.

In vitro studies addressing the effects on lipid peroxidation of β-carotene alone or in combination with α-tocopherol yielded conflicting results. Whereas two studies failed to show a protective role of β-carotene against LDL oxidation,35,36 Jialal et al.37 reported an efficient inhibition of LDL oxidation by β-carotene after loading of LDL with significant amounts of β-carotene. The molar concentrations of β-carotene used were higher than those found in healthy subjects17,30 and both in patients and in controls of this study. Whereas the LDL samples of our study patients contained on average 8 α-tocopherol molecules per particle, β-carotene was present only in small amounts, 0.02 molecules per particle before and 0.31 molecules per particle after supplementation. This indicates that, as found for healthy individuals,17,30 only about one third of LDL particles contained a β-carotene molecule, whereas others did not. However, also under this condition, β-carotene supplementation increased the LDL resistance to oxidation in 81% of patients.

In healthy subjects taking an antioxidant preparation containing α-tocopherol, β-carotene, vitamin C, and zinc changes in lag times ranged from about −20% to +85% of baseline values. Reaven et al.3 applied β-carotene alone or in combination with either α-tocopherol or α-tocopherol plus vitamin C to healthy subjects during subsequent periods, leading to plasma concentrations up to about 5 times normal values for α-tocopherol and about 20 times normal values for β-carotene. No effects on different parameters of lipid peroxidation were observed when β-carotene was given alone. In view of the extremely high increase in LDL β-carotene values, the question arises if at this concentration the same effects can be expected as within physiological ranges. Jialal et al.4 evaluated a combined antioxidant preparation of α-tocopherol, β-carotene, and ascorbate vs. α-tocopherol alone in healthy volunteers and found no significant differences in the effects on LDL oxidation kinetics. In another study,1 vitamin E sufficient smokers with a marginal β-carotene status supplemented with β-carotene, exhibited a 16.6-fold increase in LDL β-carotene concentrations and a significant prolongation of lag times, but differences in lag times after supplementation between treatment and placebo group as well as those in changes of lag times between the two groups were not significant. Increases in lag times were 41% after vitamin E, but only 6% after β-carotene supplementation. Whereas this study on subjects with marginal pretreatment values showed only a small effect of β-carotene on LDL resistance to oxidation, vitamin E-sufficient but severely β-carotene-deficient CF patients enrolled in our study exhibited a 38% increase in lag times during correction of β-carotene deficiency and reached values of healthy controls. The fact that these patients exhibited severe β-carotene deficiency at study entry, whereas participants of other studies had normal or marginal values before supplementation, may explain the more pronounced effect of β-carotene supplementation on the LDL resistance to oxidation.

The response to oral β-carotene supplementation of β-carotene concentrations in plasma and LDL of patients was characterized by a substantial between-subject variation, as was the case for the effect of β-carotene on both lag times and plasma MDA concentrations, resulting in a lack of statistical relation between β-carotene increase and changes in lag times and plasma MDA concentrations. A similar observation has already been made in healthy subjects supplemented with α-tocopherol,29 leading to the conclusion that its efficiency is subject-specific and can be characterized by the slope of the regression line of lag time on LDL α-tocopherol concentrations measured at different time points during supplementation. In this study, this observation is extended for β-carotene by the highly individual but overall significant response in lag times and plasma MDA values to correction of β-carotene deficiency. The reasons why the efficiency of β-carotene shows this high subject-specificity are not yet clear, but several factors might be involved. Not only could the fatty acid content of LDL significantly determine the length of lag times and other parameters of LDL oxidation,23,38–41 as could other plasma and tissue lipids affect the formation of MDA, but also the efficiency of antioxidants added during supplementation could highly depend on the amount of unsaturated lipids to be protected. If this holds true for healthy individuals, it is supposed to be even more the case in a patient population with documented variability in the fatty acid status.42 In
addition, other antioxidants such as ubiquinol\textsuperscript{13} and structural characteristics of the LDL particle\textsuperscript{14} have been demonstrated to determine the LDL susceptibility to oxidation. During the last years much less information has been obtained on factors contributing to MDA formation. However, the oxidative stress status of an individual could not only determine the extent of lipid peroxidation taking place in tissues and organs but also the efficiency of antioxidant supplements, particularly in patients exhibiting increased formation of reactive oxygen species due to specific disease processes.

The study patients were clinically stable, and no modifications other than \textbeta-carotene supplementation were introduced during the study period. Particularly, neither any interventions in the diet nor in the treatment of fat malabsorption were made which could have led to changes in the fatty acid status and a subsequent effect on the LDL resistance to oxidation.\textsuperscript{23,38-41} Published data on healthy subjects\textsuperscript{27} document that the LDL resistance to oxidation determined by the assay used in this study is fairly constant over several weeks in healthy subjects,\textsuperscript{27} as do our own unpublished data for CF patients if the antioxidant status does not change. Therefore, the changes observed in this study can reliably be attributed to changes in the \textbeta-carotene status.

No changes in the maximum rate of oxidation were observed during \textbeta-carotene supplementation in our study, and patients had similar rates of oxidation compared with controls. This finding is in agreement with that of Abbey et al.\textsuperscript{2} for supplementation with a combined antioxidant preparation and with that of Princen et al.\textsuperscript{1} for \textbeta-carotene supplementation, but contrasts to the observation of a significant decline in the rate of oxidation during \alpha-tocopherol supplementation.\textsuperscript{1} The prolongation of lag times by \textbeta-carotene without reduction of the rate of oxidation and of maximum diene formation could be explained by the concept of an inhibitory action of \textbeta-carotene on the initiation of lipid peroxidation, during which the antioxidant is consumed and would thus not exert any further effect on the propagation of lipid peroxidation.

In addition, this study documents a 1.5-fold higher MDA formation in CF patients with a normalized vitamin E status, but uncorrected \textbeta-carotene deficiency compared with healthy controls, which is in line with preliminary data from another group.\textsuperscript{5,6} In the presence of a normalization of plasma \textbeta-carotene concentrations, plasma MDA concentrations decreased to values comparable to those of controls. Also in healthy volunteers, depleted from \textbeta-carotene during 2 week dietary restriction, a 4 month \textbeta-carotene repletion with either 15 or 120 mg daily was associated with a significant decline in MDA concentrations.\textsuperscript{7} Although the group on the higher dosage achieved about 2.6-fold higher \textbeta-carotene concentrations than the other group after having had similar values before supplementation, no differences in the effects on lipid peroxides were observed. These findings confirm the absence of correlation between changes in plasma \textbeta-carotene levels and plasma MDA concentrations observed in this study, suggesting that either the dosage required for a comparable effect may differ substantially between individuals, as could be concluded from the data of this study, or that above a certain threshold level a further protective effect will not be observed. Obviously, much work remains to be done to answer these open questions in order to determine the most efficient antioxidant supplementation for different individuals.

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β-carotene and lipid peroxidation in CF