EFFECTS OF UBIQUINOL-10 AND β-CAROTENE ON THE IN VITRO SUSCEPTIBILITY OF LOW-DENSITY LIPOPROTEIN TO COPPER-INDUCED OXIDATION

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ABSTRACT

**Background:** Dietary antioxidant intake has been reported to be inversely associated with coronary artery disease. To clarify the possible role of lipophilic antioxidants in the prevention of atherosclerosis, we investigated the effects of ubiquinol-10 and β-carotene on the susceptibility of low-density lipoprotein (LDL) to oxidative modification.

**Methods:** In this study, first “ubiquinol-10 and β-carotene” were added to plasma and incubated for 3hr at 37°C. Then, the LDL fraction was separated by ultracentrifugation. The oxidizability of LDL was estimated by measuring conjugated diene (CD), lipid peroxides and thiobarbituric acid-reactive substances (TBARS) after cupric sulfate solution was added.

**Results:** We showed that ubiquinone-10 and β-carotene significantly (p<0.01 by ANOVA) and dose-dependently prolonged the lag time before initiation of oxidation reaction. Also, these two compounds suppressed the formation of lipid peroxides and TBARS more markedly than others. The ability of them to prolong lag time and suppression of lipid peroxides and TBARS formation resulted to be in the following order: ubiquinol-10> β-carotene.

**Conclusion:** LDL exposed to the lipophilic antioxidants in vitro reduced oxidizability. These findings suggest that ubiquinol-10 and β-carotene have a role in ameliorating atherosclerosis.


Keywords: LDL, Oxidation, Atherosclerosis, Ubiquinol-10, β-Carotene.

INTRODUCTION

Oxidation of low-density lipoprotein (LDL) has been suggested as a causal factor in human atherosclerosis. Oxidatively modified LDL is a potent ligand for scavenger receptors on macrophages and thus contributes to the generation of macrophage-derived foam cells, the hallmark of early atherosclerotic fatty streak lesions. Many additional mechanisms by which oxidized LDL may contribute to atherosclerosis have been identified. The oxidative modification hypothesis of atherosclerosis is supported by numerous in vivo findings, e.g., the presence of epitopes of oxidatively modified LDL in atherosclerotic lesions and elevated titers of circulating autoantibodies against oxidized LDL in patients with carotid atherosclerosis. Antioxidant compounds provide resistance to this process and have been suggested to pos-
sessed lower atherogenicity. Considerable epidemiologic, biochemical, and clinical evidence has accumulated in support of this hypothesis.

LDL contains different lipophilic antioxidants, the most abundant being β-tocopherol that is the major form of vitamin E. All the other antioxidants (β-tocopherol, carotenoids, ubiquinol-10) are much less present in LDL.

Vitamin E is thought to be the major nonenzymatic antioxidant present in the lipid structures of cells and lipoproteins. It is a donor antioxidant (reductant), which increases the LDL resistance against the oxidative modification. In lipid solutions and dispersions, it inhibits radical formation linearly with time until consumed in the process.

Ubiquinol-10, the reduced form of ubiquinone-10 (co-enzyme Q10), is a well-known proton-electron carrier in the inner mitochondrial membrane and a potent lipophilic antioxidant in different cell membranes and LDL. It is well established that both ubiquinols and ubiquinones are active against lipid peroxidation in mitochondria and liposomes but that the quinols are much more powerful antioxidants than the corresponding quinines.

Recently, it has been suggested that ubiquinol-10 can protect human LDL more efficiently against lipid peroxidation than vitamin E, even though it is present in LDL in much lower concentrations. Yalcin et al. reported on the prominent antioxidant activity of ubiquinol-10 in human LDL. They showed that ubiquinol-10 is the antioxidant first consumed during in vitro LDL oxidation. Also, it has been suggested by Tribble et al. that the ubiquinol-10 level may represent a surrogate measure of some other LDL property affecting its oxidizability, for example, its initial lipid hydroperoxide content.

Carotenoids have been accepted to play a preventive role in a variety of diseases, cancer and aging. β-Carotene has been selected as a function as a precursor of retinal and retinoic acid, quencher of electronically excited species such as a singlet oxygen and triplet sensitizer, and antioxidant in tissue and plasma. The inhibition by β-carotene of photosensitized oxidants of lipids mediated by singlet oxygen has been clearly demonstrated. Burton and Ingold found that β-carotene inhibited the peroxyl-radical-mediated oxidants of tetralin and methyl linolate in solution. Such antioxidant action of β-carotene has also been observed in the oxidations of lipids in solution.

In this study, we investigated the effects of plasma preincubation with ubiquinol-10 and β-carotene on the susceptibility of LDL to oxidative modification.

**MATERIAL AND METHODS**

**Chemicals**

Ubiquinol-10, β-carotene and other reagents were purchased from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

**In vitro addition of ubiquinol-10, β-carotene to plasma**

Blood was collected in an ethylenediaminetetraacetic acid (EDTA)-containing (1g/L) tube from a normal lipidemic healthy male volunteer after an overnight fast. Plasma was separated by low-speed centrifugation at 1000g at 4°C for 15 min. To enrich LDL with lipophilic antioxidants, each one of two compounds – dissolved in 10% dimethyl sulfoxide (DMSO) in phosphate buffered saline (PBS, pH=7.4) (20mL 10% DMSO/L plasma) – were added to plasma (50, 100 and 200 μmol/L) and then incubated at 37°C for 3 hr.

**Isolation of LDL**

LDL (density 1.019-1.063 g/mL) was isolated from the plasma in the 1.019-1.063 g/mL density range by a rapid isolation technique as reported by Chung et al. The pooled plasma solvent density was adjusted to 1.019 g/mL with a high density salt solution (containing NaCl, KBr, and EDTA). After centrifugation for 1 hr at 100,000g and 16°C, the top layer of supernatant was removed. The density of pooled infranatant was adjusted to 1.063 g/mL and was recentrifuged as before. The LDL, which floats at a relative density of 1.063, was collected and exhaustively dialyzed for 12 hr at 4°C with four 1-L changes in EDTA-free PBS (pH=7.4) that had been degassed by using a vacuum procedure. Control LDL was prepared by the same technique after adding only vehicle (20 min 10% DMSO/L plasma) to the plasma and incubating for 3 hr at 37°C. In a preliminary study, we confirmed that DMSO at this concentration did not affect the oxidizability of LDL. The LDL suspension was stored at 4°C under argon in the dark.

**Measurement of oxidizability of LDL**

The oxidizability of LDL was estimated by measuring three indexes including: conjugated dienes (CD), lipid peroxides, and thiobarbituric acid-reactive substances (TBARS).

1) CD: Freshly prepared LDL (50 mg protein/L) was incubated with cupric sulphate solution (final concentration 10 μmol/L) at 37°C in a Hitachi spectrophotometer (Hitachi Sangyo Co, Tokyo) fitted with a peltier heater. The increase in absorbance at 234 nm was automatically recorded at 10-min intervals. Lag time before the initiation of oxidation and the propagation rate were determined according to the methods of Esterbauer et al with some modification. The output from the spectrophotometer was converted directly into the ASCII file.
format and used to compute the lag time prior to the onset of oxidation. The spectrophotometer was converted directly into the ASCII file format and used to compute the lag time prior to the onset time of maximum diene concentration, which was determined from the difference between the absorbance curve and the absorbance at time zero using the extinction coefficient for conjugated dienes at 234 nm (E=295,000 mol/cm$^3$). After incubation for 90 or 180 min with 10 μmol CuSO$_4$/L, the oxidation reaction was stopped by adding EDTA (100 μmol/L final concentration).

2) Lipid peroxides: The content of lipid peroxides in LDL was measured colorimetrically with a commercially available kit. For measuring the content of lipid peroxides in LDL, we used an assay based on the oxidative activity of LOOH, which convert iodide to iodine. Iodine is further to form the triiodide ion, which absorbs at 365 nm. We used the microtitre plate procedure by using a commercially available reagent (CHOD-iodide, Merck, Darmstadt, Germany). The LDL oxidation was carried out in polystyrene microtitre plates. 190 μL of room-tempered CHOD iodide reagent was added to plates and incubated for 60 min at 37°C. The absorbance at 365 nm was measured in a microplate reader (Labsystems, Muluskan MCC/340, Uppsala, Sweden). The concentration of LOOH was calculated from the molar absorption coefficient of 246,000 mol/cm$^3$ for the triiodide ion and a path length in the microtitre plate of 1 cm for the final volume of 330 μL.

3) TBARS: The concentration of TBARS was also measured by using the method described by Buege and Aust. LDL (0.1 mL) was mixed with 1 mL of 0.67% TBA and 0.5 mL 20% trichloroacetic acid and incubated at 100°C for 20 min. After cooling, the reaction mixture was centrifuged at 4000 rpm for 5 min and the absorbance of the supernatant read at 532 nm. The concentration of TBARS was calculated by using the extinction coefficient of 165,000 mol/cm. The concentration of TBARS was expressed as nmol of malondialdehyde (MDA) equivalents per mg LDL protein using a freshly diluted 1,1,3,3-tetraethoxypropane for the standard curve.

**Statistical analysis**

All results are presented as mean ± standard deviations (n= 5). Data between groups were compared by analysis of variance (ANOVA). Fisher’s test was used whenever a statistically significant difference between the two groups was shown by ANOVA.

**RESULTS**

Addition of ubiquinol-10 and β-carotene to LDL for 3 hr at 37°C resulted in lipoprotein antioxidants enrichment. Of course, efficiency of the enrichment depended on the compound concentration in medium.

Incubation of copper ions with the LDL suspension caused extensive oxidation of the lipoprotein as judged by measuring of CD, lipid peroxides and TBARS in the LDL sample. Enriching LDL with antioxidant agents made it more resistant to copper-induced oxidation in comparison with a native. This effect was demonstrated with all the indices of oxidation used and appeared to be most pronounced within the first hours of oxidation.

Table 1 shows the effects of ubiquinol-10 and β-carotene on the susceptibility of LDL to copper-induced oxidation by measuring of lag time. These significantly increased lag time before the onset of CD formation (p<0.01 by ANOVA). Prolongation of the the lag time by these components was dose-dependent. Continuous registration of absorbance of the LDL sample at 234 nm showed that ubiquinol-10 (200 μmol/L) exerted the strongest effects, prolonging lag time to more than three times that of the control. Also, β-carotene prolonged lag time to twice that of the control, respectively. The lag time was significantly increased in the LDL separated from pre-treated plasma with 50 μmol/L ubiquinol-10, or with 100 μmol/L ubiquinol-10 and β-carotene, or with 200 μmol/L

<table>
<thead>
<tr>
<th>Compound concentration</th>
<th>0 (control)</th>
<th>50 μmol/L</th>
<th>100 μmol/L</th>
<th>200 μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Carotene</td>
<td>58.1 ± 3.0</td>
<td>59.1 ± 6.6</td>
<td>90.0 ± 8.8</td>
<td>131.9 ± 14.0</td>
</tr>
<tr>
<td>Ubiquinol-10</td>
<td>58.1 ± 3.0</td>
<td>69.9 ± 4.9</td>
<td>91.2 ± 5.8</td>
<td>176.0 ± 9.8</td>
</tr>
</tbody>
</table>

* Mean ± SD of three separate experiments. Lag times for ubiquinol-10 and β-carotene differ significantly than control. p<0.01(ANOVA).

Significantly different from control (Fisher’s test): *p<0.01 & **p<0.05.

After adding only vehicle (20 min 10% DMSO/L plasma) to the plasma and incubating for 3 hr at 37°C, control LDL was prepared. DMSO at this concentration did not affect the oxidizability of LDL.
Effects of Ubiquinol-10 & β-Carotene on LDL Oxidation

**Table II**: Effect of addition of β-carotene & ubiquinol-10 (0-200 μmol/L) to plasma on susceptibility of low-density lipoprotein to Cu-induced oxidation by measuring of lipid peroxides.*

<table>
<thead>
<tr>
<th>Compound concentration</th>
<th>Lipid peroxides (nmol/mg protein)</th>
<th>0 (control)</th>
<th>50 μmol/L</th>
<th>100 μmol/L</th>
<th>200 μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Carotene</td>
<td>214.0 ± 2.0</td>
<td>197.52 ± 4.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>129.44 ± 14.54b</td>
<td>59.83 ± 10.62b</td>
<td></td>
</tr>
<tr>
<td>Ubiquinol-10</td>
<td>214.0 ± 2.0</td>
<td>196.0 ± 5.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.33 ± 7.15b</td>
<td>25.90 ± 8.20b</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SD; n=4. One representative experiment of three is shown; the other two experiments yielded similar results.

Lipid peroxides differ significantly between groups, *p*<0.01 (ANOVA).

<sup>a</sup>Significantly different from control: *p*<0.05 & *p*<0.0001.

After adding only vehicle (20 min 10% DMSO/L plasma) to the plasma and incubating for 3 hr at 37°C, control LDL was prepared. DMSO at this concentration did not affect the oxidizability of LDL.

**Table III**: Effect of addition of β-carotene and ubiquinol-10 (0-200 μmol/L) to plasma on susceptibility of low-density lipoprotein to Cu-induced by measuring of TBARS.*

<table>
<thead>
<tr>
<th>Compound concentration</th>
<th>TBARS (nmol MDA/mg protein)</th>
<th>0 (control)</th>
<th>50 μmol/L</th>
<th>100 μmol/L</th>
<th>200 μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Carotene</td>
<td>15.60 ± 1.60</td>
<td>9.51 ± 0.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.39 ± 0.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.89 ± 1.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Ubiquinol-10</td>
<td>15.60 ± 1.60</td>
<td>9.42 ± 0.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.62 ± 0.93&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.23 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

*Mean ± SD; n=4. One representative experiment of three is shown; the other two experiments yielded similar results.

TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde. TBARS differ significantly between groups, *p*<0.01 (ANOVA).

<sup>a</sup>Significantly different from control: *p*<0.05 & *p*<0.0001.

After adding only vehicle (20 min 10% DMSO/L plasma) to the plasma and incubating for 3 hr at 37°C, control LDL was prepared. DMSO at this concentration did not affect the oxidizability of LDL.

ubiquinol-10 and β-carotene (*p*<0.01 & *p*<0.05 by Fisher’s test). The capacity to prolong the lag time was in the following order: ubiquinol-10>β-carotene.

Suppression of TBARS and lipid peroxides formation was observed in the LDL samples to which these agents had been added. Tables II and III show results obtained. In these experiments, ubiquinol-10 exerted a stronger effect and suppressed the formation of TBARS and lipid peroxides more markedly than β-carotene. Effects were proportional to the compound doses. TBARS and lipid peroxide formation in the medium was significantly reduced in the LDL separated from plasma that had been incubated with 50, 100 and 200 μmol/L ubiquinol-10 and β-carotene (*p*<0.05 & *p*<0.0001). The suppression ability of TBARS and lipid peroxide formation was in the following order: ubiquinol-10>β-carotene.

**DISCUSSION**

In this study we showed that the copper-catalyzed oxidation of LDL, isolated from plasma preincubated with ubiquinol-10 and β-carotene, was significantly inhibited in a dose-dependent manner as assessed by lag time before the initiation of oxidation and the formation of TBARS and lipid peroxides. To add these two compounds to LDL in vitro, we incubated EDTA-containing plasma at 37°C for 3 hr with each one of them at the concentrations 50, 100 and 200 μmol/L; LDL was then separated and dialyzed against PBS before the induction of oxidation. Therefore, the compounds not associ-
ated with LDL particles were removed during dialysis. Thus, they that were on the surface of or within LDL particles were considered to be responsible for inhibiting LDL oxidation.

The concentration of both compounds chosen for this study was obtained from our previous dose-response studies and the published data and is equivalent to values in the upper end of the reference range.\textsuperscript{22,23}

Ubiquinol-10 and β-carotene are known to be highly efficient lipid-soluble antioxidants in the protection of lipids in a variety of biological and model systems including cell membranes, LDL and liposomes. Ubiquinol-10 is generally assumed that the quinol may exhibit its protective effect by preventing the formation of lipid free radicals and/or by eliminating them. On the other hand, the possibility that the inhibition of LDL modification such as oxidation or glycation by ubiquinol-10 is related to its interactions on the lipid structure of the LDL particle needs further investigation.\textsuperscript{24}

Ubiquinol-10 and β-carotene contain shielding methyl(-CH\textsubscript{3}) and methoxyl(-OCH\textsubscript{3}) groups adjacent to the phenolic hydroxyl (-OH) groups in the LDL particle by its phytol side-chain. In addition, both protect LDL against peroxidative modification, maintaining its ability to act as a ligand for LDL receptors.\textsuperscript{25,26}

Moreover, \textit{in vivo} supplementation of LDL with ubiquinol-10 is known to decrease LDL susceptibility to modification and to increase LDL clearance in the plasma. A decreased level of total ubiquinol is found in plasma of patients with atherosclerosis as well as numerous examples of successful treatment with ubiquinol-10 of some pathologies raise the possibility that ubiquinol-10 may decrease the effect of LDL in formation of atherosclerotic lesions.\textsuperscript{16} β-Carotene differs from the other major fat-soluble antioxidant, vitamin E, in being more effective at low oxygen pressures. However, in concert with the tocopherols, it is strategically carried primarily in the LDL particle and hence affords LDL with a defense against oxidative attack.\textsuperscript{17} Currently, there is much intensive effort being directed at elucidating β-carotene’s postulated cancer chemo-preventative properties. However, as an antioxidant, β-carotene might also have a role in preventing the development of the atherosclerotic lesion. Previously, it has been shown that smoking, as a major risk factor for coronary artery disease, results in decreased levels of both ascorbic acid and β-carotene.\textsuperscript{27}

This antioxidant deficiency could render the LDLs of smokers more prone to oxidative modification and hence promote atherogenesis. Also, β-carotene can retard the progression of atherosclerosis in LDL-receptor-deficient rabbits. To this end, it recently was reported in preliminary form that β-carotene supplementation significantly reduced not only major coronary events but all major vascular events in a group of man subjects. It is tempting to speculate that this beneficial effect of β-carotene was being mediated in part by its inhibitory effect on LDL oxidation.\textsuperscript{28}

Incubation of both compounds to plasma over a long period of time may enrich the LDL particles sufficiently to make them less susceptible to oxidative reaction.

In conclusion, we clearly showed that incubation of plasma with ubiquinol-10 and β-carotene protected LDL from copper-induced oxidation reaction. These compounds significantly decreased the susceptibility of LDL to oxidative modification; therefore, they may have favorable effects in ameliorating atherosclerosis.

\textbf{REFERENCES}