Effects of Antioxidants on the Oxidative Susceptibility of Low-Density Lipoprotein

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Summary An important event in the pathogenesis of atherosclerosis is believed to be the oxidative modification of low-density lipoprotein (LDL) initiated by a free radical-driven lipid peroxidation process. Vitamin E acts as a lipophilic chain-breaking antioxidant, while water-soluble chain-breaking antioxidants such as vitamin C or uric acid suppress the oxidation of LDL initiated by aqueous radicals. In this study, we established a new method of measuring the lag time of inhibited lipid peroxidation using the lipophilic azo radical initiator V-70: 2-2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) and investigated in vitro the susceptibility of LDL to oxidation using this method when lipid- and water-soluble antioxidants were added. When the lipid-soluble antioxidant, vitamin E, was added to LDL, the lag time was extended whereas a higher dose of vitamin E led to a shortened lag time of V-70-induced lipid peroxidation in LDL. These results suggest that vitamin E radicals (tocopheroxyl radicals) act as prooxidants during the autoxidation of LDL. It was also shown that the shortened lag time induced by higher doses of vitamin E was restored when lipid- and water-soluble antioxidants were added simultaneously, which suggests that vitamin E radicals derived from vitamin E are subsequently reduced by vitamin C to regenerate vitamin E. Thus, the interaction between lipid- and water-soluble antioxidants provides an important function in maintaining LDL resistance to oxidation.

Key Words atherosclerosis, antioxidant, vitamin E, vitamin C, oxidized LDL

The oxidation of low-density lipoprotein (LDL) is considered to be an important risk factor of the atherogenic progression in cardiovascular disease (1). Oxidized LDL can attract monocytes into the arterial wall where they can be transformed

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into macrophages—the precursors of foam cells. It also immobilizes macrophages, which causes oxidized LDL to be retained in the arterial wall and, finally, it can be cytotoxic and produce injury to endothelial cells and smooth muscle cells (2-5). Thus, multiple mechanisms occurring within the arterial wall may promote LDL oxidation. The inhibition of LDL oxidation should therefore reduce the formation of atherosclerotic lesions.

The free radical-mediated oxidation of LDL proceeds to lipid peroxidation, which is the autoxidation of the polyunsaturated fatty acid chains (PUFA) of lipids by a radical chain reaction (6). The role of chain-breaking antioxidants is quite important, since they scavenge chain-carrying radicals consequently breaking the chain reaction. In fact, the endogenous antioxidants of human plasma and LDL can prevent lipid peroxidation by radical trapping defense systems (7, 8). Vitamin E, a constituent of LDL, acts as a lipophilic chain-breaking antioxidant and water-soluble antioxidants in plasma such as vitamin C and uric acid play a similar role as vitamin E or other lipophilic antioxidants within the LDL particles. A compilation of lipid- and water-soluble antioxidants suppresses the oxidation of LDL.

Esterbauer et al. (9) developed a method to assess the susceptibility of LDL to copper-mediated oxidation in vitro by continuous monitoring of the formation of conjugated dienes. When LDL is exposed to oxidative conditions (Cu^{2+}, cultured cells, azo compounds), a lag time precedes the oxidation of PUFAs. The lag time is an index of the oxidation resistance to LDL.

In the oxidation process of LDL mediated by Cu^{2+}, the LDL solution must be dialyzed extensively to remove chelation reagents like EDTA prior to the oxidation of LDL. However, prolonged dialysis of LDL may result in the loss of smaller molecular weight components that have antioxidant properties, affecting the true outcome of measuring the copper-initiated oxidation of LDL (10).

The aim of this study was to investigate the in vitro susceptibility of LDL to oxidation by the addition of lipid- and water-soluble antioxidants using a new method of measuring conjugated dienes triggered by V-70 (2-2'-azobis(4-methoxy-2,4-dimethylvaleronitrile)), called the azo radical-initiator (11).

MATERIALS AND METHODS

Isolation and preparation of LDL. Blood samples were collected in EDTA-containing tubes from healthy normolipidemic adult volunteers after an overnight fast. Plasma was prepared by centrifugation at 3,000 rpm (2,000 g) for 10 min at 4°C. LDL was isolated from 1.1 mL plasma by single-spin density gradient ultracentrifugation (100,000 rpm (417,000 g), 40 min, 4°C) with a TLA-100.4 fixed-angle rotor (Beckman, Palo Alto, CA). The density of the plasma was adjusted to \( d = 1.21 \) g/mL by adding 0.3575 g of KBr to 3.9 mL-volume ultracentrifuge tubes and then filling the air space of the tubes with about 2.8 mL of \( d = 1.006 \) g/mL saline-EDTA buffer (12).
The protein content of LDL was determined by bicinchonic acid (BCA) assay using bovine albumin as the standard. Before the start of the oxidation experiments, LDL samples were diluted with PBS to give final concentrations of 70 µg/mL LDL protein.

**LDL oxidation.** Oxidation was initiated by the addition of freshly prepared V-70: 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (Wako Pure Chemical, Osaka, Japan) solution at final concentrations of 200 µM. The kinetics of the oxidation of LDL were determined by monitoring the change of conjugated diene formation at 234-nm absorbance by a Beckman Model DU 650 spectrophotometer equipped with a 12-position automatic sample changer, and the change at 234-nm in absorbance was recorded at 5 min intervals at 37°C.

The absorbance curve at 234-nm was divided into three phases; lag time, propagation phase and decomposition phase. The lag time was defined as the interval between the intercept of the tangent of the slope of the curve in the propagation phase with the baseline, and was expressed in minutes.

**In vitro antioxidant addition to LDL.** We measured the oxidation lag time of human LDL on the condition that the final concentration of LDL-protein and V-70 was 70 µg/mL protein and 200 µM, respectively. In this experiment, it was confirmed that the structure of vitamin E and uric acid influenced absorbance at 234 nm. We prepared a final concentration of LDL-protein and V-70 as follows for the purpose of monitoring all the process of LDL oxidation.

The lag time was measured after vitamin C, L(+)-ascorbic acid (Wako Pure Chemical) was added at final concentrations of 0.07, 0.14, 0.35, 0.7 and 1.4 mg/dL followed by V-70 at a final concentration of 200 µM to a preparation of LDL (70 µg/mL protein). Uric acid (Sigma, St. Louis, MO) was added at final concentrations of 0.2, 0.4 and 0.6 mg/dL and V-70 at a final concentration of 400 µM.

Vitamin E, RRR-α-tocopherol, which is the natural isomer (Eisai, Tokyo, Japan) used as a lipid-soluble antioxidant, was added to LDL (35 µg/mL protein) at final concentrations of 0.1, 0.5, 1.0, 1.5, 3.0 and 6.0 mg/dL, and samples were preincubated at 37°C for 10 min to incorporate vitamin E into LDL before adding V-70 at a final concentration of 200 µM. The lag time was then measured to assess the resistance of LDL to oxidation.

**RESULTS**

Figures 1 and 2 describe the production of conjugated dienes when the water-soluble antioxidants, vitamin C and uric acid, were added to LDL, respectively.

Vitamin C prolonged the lag time (Fig. 1) in a dose-dependent manner. Compared with the control, the lag time was prolonged 1.3 times in the presence of 0.07 mg/dL vitamin C, 1.7 times at 0.14 mg/dL, 3.4 times at 0.35 mg/dL and 4.1 times at 0.7 mg/dL.

Similar results were observed with uric acid. Figure 2 shows that the lag time
Fig. 1. Inhibition of conjugated diene formation in the oxidation of LDL (70 μg/mL LDL protein) by vitamin C at 37°C. The oxidation was initiated by the addition of 200 μM (final concentration) V-70. The final concentrations of vitamin C were: □, 0; ●, 0.07; △, 0.14; ▲, 0.35; ◊, 0.7; and ◆, 1.4 mg/dL.

Fig. 2. Inhibition of conjugated diene formation in the oxidation of LDL (70 μg/mL LDL protein) by uric acid at 37°C. The oxidation was initiated by the addition of 400 μM (final concentration) V-70. The final concentrations of uric acid were: □, 0; ●, 0.2; △, 0.4; and ▲, 0.6 mg/dL.

was prolonged dose-dependently and a propagation phase was not formed in the presence of 0.6 mg/dL uric acid during 390 min of incubation. Since the normal concentration of uric acid in plasma ranges between 4.5 and 5.5 mg/dL, the oxidative modification of LDL in vivo is apparently well controlled by the physiological levels of plasma uric acid.

J Nutr Sci Vitaminol
Effects of Antioxidants on Low-Density Lipoprotein

Table 1. Lag time of conjugated diene formation in LDL (35 µg/mL LDL protein) in the oxidation induced by 200 µM (final concentration) V-70.

<table>
<thead>
<tr>
<th>Vitamin E addition (mg/dL)</th>
<th>Lag time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>32.5</td>
</tr>
<tr>
<td>0.1</td>
<td>66.2</td>
</tr>
<tr>
<td>0.5</td>
<td>106.3</td>
</tr>
<tr>
<td>1.0</td>
<td>108.3</td>
</tr>
<tr>
<td>1.5</td>
<td>186.2</td>
</tr>
<tr>
<td>3.0</td>
<td>237.7</td>
</tr>
<tr>
<td>6.0</td>
<td>148.1</td>
</tr>
</tbody>
</table>

The suspensions of LDL and vitamin E were incubated for 10 min at 37°C prior to the addition of 200 µM (final concentration) V-70.

Table 2. Lag time of conjugated diene formation in LDL (35 µg/mL LDL protein) in the oxidation induced by 200 µM (final concentration) V-70.

<table>
<thead>
<tr>
<th>Probucol addition (mg/dL)</th>
<th>Lag time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>42.5</td>
</tr>
<tr>
<td>0.2</td>
<td>136.5</td>
</tr>
<tr>
<td>0.4</td>
<td>185.2</td>
</tr>
<tr>
<td>1.0</td>
<td>245.3</td>
</tr>
<tr>
<td>2.0</td>
<td>124.2</td>
</tr>
<tr>
<td>4.0</td>
<td>86.6</td>
</tr>
</tbody>
</table>

The suspensions of LDL and probucol were incubated for 10 min at 37°C prior to the addition of 200 µM (final concentration) V-70.

Each dose of vitamin E added to LDL was incorporated into LDL during incubation at 37°C for 10 min (data not shown). Following incorporation of the vitamin E into LDL, the lag time was extended dose-dependently up to a final concentration of 3 mg/dL (237.7 min) (Table 1). However, the addition of 6 mg/dL of vitamin E led to a shortening of the lag time (148.1 min).

The antioxidant probucol, a drug used in the treatment of hypercholesterolemia, acted similarly to vitamin E. The lag time was extended dose-dependently by the addition of 1 mg/dL probucol, though 2 mg/dL probucol shortened the lag time (Table 2).

Finally, we investigated the interactive effects of lipid- and water-soluble antioxidants on LDL oxidative susceptibility. Figure 3 and Table 3 show that the lag time which was shortened by the addition of high concentrations of vitamin E was reversed by adding vitamin C and uric acid. The interaction of vitamin E with vitamin C extended the lag time as compared to the control (33 min). The lag time was 124.9 min after adding vitamin E (at a final level of 6 mg/dL) and prolonged...
Fig. 3. Effect of vitamin C on furthering the extension of lag time of conjugated diene formation in the oxidation of LDL (35 μg/mL LDL protein) by vitamin E. The oxidation was initiated by the addition of 200 μM (final concentration) V-70. □, without antioxidants; ●, with 6 mg/dL vitamin E; △, with 6 mg/dL vitamin E and 0.5 mg/dL vitamin C; ▲, with 6 mg/dL vitamin E and 1 mg/dL vitamin C.

Table 3. Effect of uric acid on the further extension of lag time of conjugated diene formation in the oxidation of LDL (70 μg/mL LDL protein) by vitamin E.

<table>
<thead>
<tr>
<th>Addition (mg/dL)</th>
<th>Lag time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>28.0</td>
</tr>
<tr>
<td>Uric acid 0.2</td>
<td>100.6</td>
</tr>
<tr>
<td>Uric acid 0.4</td>
<td>304.9</td>
</tr>
<tr>
<td>Vitamin E 1.5</td>
<td>169.4</td>
</tr>
<tr>
<td>Vitamin E 1.5 + Uric acid 0.2</td>
<td>262.4</td>
</tr>
<tr>
<td>Vitamin E 1.5 + Uric acid 0.4</td>
<td>&gt;420.0</td>
</tr>
<tr>
<td>Vitamin E 3.0</td>
<td>74.5</td>
</tr>
<tr>
<td>Vitamin E 3.0 + Uric acid 0.2</td>
<td>277.1</td>
</tr>
<tr>
<td>Vitamin E 3.0 + Uric acid 0.4</td>
<td>&gt;420.0</td>
</tr>
</tbody>
</table>

The oxidation was initiated by the addition of 400 μM (final concentration) V-70. The suspensions of LDL and vitamin E were incubated for 10 min at 37°C prior to the addition of uric acid and V-70.

to 324.2 min after adding vitamin C.

There was a dose-dependent effect of uric acid on LDL oxidizability. As shown in Table 2, the lag time in the presence of 0.2 mg/dL uric acid was 100.6 min and 304.9 min at 0.4 mg/dL. In the same experiments, the lag time with 1.5 mg/dL of vitamin E was 169.4 min and 74.5 min with 3 mg/dL of vitamin E. This result differs from that shown in Table 1, because LDL used in each experiment was taken from

*J Nutr Sci Vitaminol*
individual subjects and the conditions of the experiments also differed. When vitamin E and uric acid were added together, the lag time was 262.4 min (1.5 mg/dL vitamin E and 0.2 mg/dL uric acid) or 277.1 min (3 mg/dL vitamin E and 0.2 mg/dL uric acid). Further, when the uric acid dose was increased to 0.4 mg/dL, lag times were extended longer.

**DISCUSSION**

In this study, we investigated the interactive effect of vitamin E, vitamin C and uric acid on the oxidation of LDL with our new method using a lipophilic azo radical initiator at ambient temperature (11).

Oxidative modifications of LDL proceed by a radical chain reaction. The initial step of the lipid peroxidation process follows the abstraction of a hydrogen atom (H') from a PUFA (LH) within the LDL particle, leading to formation of a carbon-centered lipid radical (L'). The carbon-centered lipid radical rapidly reacts with O_2 to form a peroxyl radical, LOO'. The lipid peroxyl radical can then react with an adjacent PUFA side chain (L'H) within LDL to form another carbon-centered lipid radical, thereby propagating the radical chain reaction. Therefore, a single initiating event can trigger many rounds of PUFA autoxidation, resulting in the formation of a large number of lipid hydroperoxides (LOOH) with conjugated dienes (13).

Aerobic organisms are protected from this oxidative damage by an array of defense systems. Vitamin E, β(beta)-carotene, ubiquinol, etc., the lipophilic chain-breaking antioxidants present within LDL particles, suppress the oxidative damage. Water-soluble chain-breaking antioxidants such as vitamin C, uric acid and albumin, and probably HDL, scavenge aqueous radicals and suppress the oxidation of LDL from the outside.

Our studies showed that the lag time was actually reduced when a high dose of vitamin E was incorporated into LDL particles. As Fig. 3 shows, the shortened lag time, however, recovered in the presence of vitamin C, which was present outside of the LDL particles. The phenomenon was caused by vitamin E which acted either as a prooxidant or antioxidant. Several reports have shown that high levels of vitamin E act as a prooxidant during the autoxidation of LH (14–16). Bowry et al suggested that peroxidation was propagated within LDL particles by the vitamin E radical through the following reactions, (1) and (2) (14).

\[
\text{TocH} + \text{LOO}^* \rightarrow \text{Toc}^* + \text{LOOH} \quad (1)
\]

\[
\text{Toc}^* + \text{LH} \rightarrow \text{TocH} + \text{L}^* \quad (2)
\]

\[
\text{TocH (vitamin E)}
\]

\[
\text{Toc}^* \text{ (vitamin E radical)}
\]

This prooxidant effect of vitamin E leads to an increase in the conjugated diene structure with the formation of a propagation phase, i.e., it results in a shortening
of the oxidation lag time in LDL. When vitamin C is added to the aqueous phase, the rate of vitamin E consumption is reduced markedly; presumably vitamin C is an effective regenerator of vitamin E from vitamin E radical (17). Our results in the interaction between vitamin E and vitamin C are supported by previous studies.

On the other hand, the lag time which was shortened by the addition of 3 mg/dL of vitamin E also recovered in the presence of uric acid in the same way as that of vitamin C. However, it has been reported elsewhere that unlike vitamin C, uric acid does not reduce vitamin E radicals in lipid dispersion (18, 19). Further studies are needed to classify this difference.

The above results suggest that in vitro water-soluble antioxidants provide complementary actions to the antioxidant activity of vitamin E directly or indirectly. It has been reported that probucol inhibits the oxidation of LDL as efficiently as vitamin E, and both are consumed independently during LDL oxidation (20); that is, without sparing vitamin E. Probucol, a combination of two butylated hydroxytoluene (BHT) molecules coupled by an “-S-C-S-” group, is a lipophilic antioxidant and previously used as a lipid lowering drug. It is carried within LDL particles in plasma and effectively suppresses the oxidative modification of LDL in a dose-dependent manner (20–24). The lag time, which was extended dose-dependently up to 1 mg/dL of probucol, was shortened at higher doses of 2 mg/dL (Table 2) for reasons which remain unclear. Probucol may act similarly in properties to vitamin E as a prooxidant, but this action has never been reported. More research is needed to clarify the mechanisms of action of probucol at higher concentrations, including identification of the probucol degradation products.

In conclusion, our study provides evidence that LDL-oxidative resistance is enhanced by the interaction of combined lipid- and water-soluble antioxidants existing within the lipid and aqueous phases, respectively. These results were obtained using a new assay method that utilizes an azo-radical initiator. Thus, the total radical trapping capacity of antioxidants is an important determinant of LDL oxidation in human plasma, an aerobic organism.

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