Effects of Various Natural Antioxidants on the Cu^{2+} -Mediated Oxidative Modification of Low Density Lipoprotein

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We have reported in our previous paper that several flavan-3-ol derivatives (tea polyphenols) inhibited the Cu^{2+} -mediated low density lipoprotein (LDL) oxidation *in vitro*. (–)-Epigallocatechin gallate (EGCG), in particular, exhibited strong inhibition.

In this study, we have compared the antioxidative effects of EGCG with those of other natural antioxidants, such as flavonols, sesaminol, curcuminoid derivatives, tocopherol analogues, and theaflavins. The antioxidative effects were monitored by conjugated diene formation in LDL which was carried out at 37°C with 5 μM CuSO_4 with or without antioxidants. Dibutyl hydroxytoluene (BHT) was used as a reference compound. The lag-time before the onset of conjugated diene formation was more than 100 min in the presence of 0.5 μM EGCG, theaflavin, myricetin, quercetin, and sesaminol. The ability to prolong the lag-time was in the order of sesaminol > quercetin > EGCG > theaflavin $\geq$ myricetin > BHT > α -tocopherol. Among the 4 tocopherol analogues used, α -tocopherol showed the strongest antioxidative activity. We have also studied the effects of EGCG, BHT, and α -tocopherol on cholesteryl ester (CE) degradation and apolipoprotein B 100 (apo B 100) fragmentation in the Cu^{2+} -mediated oxidative modification of LDL. EGCG was the most effective inhibitor of CE degradation and apo B 100 fragmentation.

Keywords low density lipoprotein (LDL); lipid peroxidation; epigallocatechin gallate; natural antioxidant; antioxidative activity; atherosclerosis

More and more evidence is accumulating that low density lipoprotein (LDL) are the principal lipoproteins susceptible to oxidative modification leading to foam cell formation. The oxidative modification of LDL is believed to occur *via* endothelial cells, smooth muscle cells, or macrophages. The LDL, once modified oxidatively, can no longer be recognized by LDL receptors but is recognized by the scavenger receptors of macrophages, leading to the formation of cholesterol deposits in foam cells.¹⁾

Oxidation of LDL includes the process in which the polyunsaturated fatty acids present in LDL are oxidized and automatically degraded to produce a variety of aldehydes and related products.²⁾ These lipid peroxidation derived aldehydic products bind to the ϵ -amino groups of lysine residues of apolipoprotein B 100 (apo B 100) and this modified LDL can be taken up easily by macrophages to form foam cells.^{1,3-5)} Using polyclonal and monoclonal antibodies directed against oxidatively modified LDL, malondialdehyde-modified LDL, and 4-hydroxynonenal-modified LDL, it was shown by immunostaining that such forms of modified LDL are in fact present in atherosclerotic plaque of hyperlipidemic rabbits.^{1,6,7)} Antibodies against malondialdehyde and 4-hydroxynonenal-conjugated proteins were also found in human and rabbit plasma.¹⁾ The role of antioxidants and antioxidative enzymes would therefore be significant, if oxidatively modified LDL contributes to atherogenesis.

LDL are known to contain various antioxidative factors such as α -tocopherol, γ -tocopherol, β -carotene, and lycopene.⁸⁾ Furthermore, it has been reported that ascorbate, not present in LDL, can preserve endogenous antioxidants in LDL and delay the onset of lipid

peroxidation of LDL *in vitro*.⁸⁾ Moreover, ascorbate supplementation has a protective role with respect to the susceptibility of LDL to peroxidation induced by acute smoking.⁹⁾ It may be possible that other naturally occurring antioxidants directly or indirectly inhibit the oxidation of LDL and suppress its apo B 100 modification.

We have reported in previous papers that flavan-3-ol derivatives in tea leaves show marked antioxidative activities in rat plasma and organs.¹⁰⁻¹²⁾ We have also shown that flavan-3-ol derivatives, in particular (–)-epigallocatechin gallate (EGCG), a major flavan-3-ol derivative in tea leaves,^{13,14)} strongly inhibits the Cu^{2+} -mediated LDL oxidation *in vitro*.¹⁵⁾ de Whalley *et al.* have reported¹⁶⁾ that flavonoids inhibit the oxidative modification of LDL by macrophages, and they can conserve the α -tocopherol in LDL, thereby delaying the onset of detectable lipid peroxidation. Osawa has reported¹⁷⁾ that sesaminol isolated from sesame seeds was the effective antioxidant in several test systems, *in vitro*, as well as *in vivo*. He also reported that tetrahydrocurcumin obtained by hydrogenation of curcumin has strong antioxidative properties.

In the present study, we have compared the antioxidative effects of EGCG, reported previously, with other natural antioxidants such as flavonols, sesaminol, curcuminoids, tocopherol analogues, theaflavin, pigments of oolong and black tea, on the Cu^{2+} -mediated oxidative modification of LDL.

MATERIALS AND METHODS

Materials EGCG was supplied by the Food Research Laboratories, Mitsui Norin Co., Ltd. (Shizuoka, Japan).

Sesaminol was extracted from sesame seeds and purified by column chromatography.¹⁷⁾ Curcumin was extracted from *Curcuma longa*. Tetrahydrocurcumin was obtained from curcumin by hydrogenation using Pd-C as the catalyst.¹⁷⁾ Tocopherol analogues were generously supplied from Eisai Co., Ltd. (Tokyo, Japan). Quercetin was obtained by the hydrolysis of rutin, which was purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). Myricetin was prepared by the hydrolysis of myricitrin, which was kindly supplied by San-Eigen FFI Co., Ltd. (Osaka, Japan). All other chemicals were of reagent grade.

Isolation and Purification of LDL LDL ($d=1.019-1.063$) was isolated by ultracentrifugation from porcine serum in the presence of 0.01% EDTA.¹⁸⁾ After isolation, it was purified by fast protein liquid chromatography (FPLC) on a Sephacryl S400HR column (600 × 16 mm i.d., Pharmacia Co.) using 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl as the eluting solvent in the presence of 1 mM EDTA. LDL thus purified was dialyzed against 10 mM phosphate-buffered saline (PBS) containing 1 μ M EDTA for 24 h. EDTA was removed by a Sephadex G-25 column (Pharmacia PD-10; bed volume, 9.1 ml) equilibrated with PBS. The protein content was measured by the Lowry method.¹⁹⁾

LDL Oxidation LDL (100 or 200 μ g protein/ml) was diluted with 10 mM PBS and incubated at 37 °C in the presence of 5 μ M CuSO₄. The oxidation was performed in the presence or absence of antioxidants and the reaction was stopped by adding EDTA (final concentration; 1 mM). After incubation, the cholesteryl ester (CE) degradation and apo B 100 fragmentation of the LDL were measured as described below. Dibutyl hydroxytoluene (BHT) was used as a reference antioxidant.

Conjugated Diene Conjugated diene formation was measured by determining the increase in absorbance at 234 nm of the LDL solution (100 μ g protein/ml) in PBS incubated with 5 μ M CuSO₄ in the presence or absence of 0.5 or 1.0 μ M antioxidant. The absorbance was measured every 10 min for 9 h using a Hitachi U-2000 spectrophotometer and the results were expressed as the increase in the absolute absorbance at 234 nm. BHT was used as a reference antioxidant.

Electrophoresis Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 3–15% gradient) was performed after the delipidation of LDL solution (200 μ g protein/ml) by the method of Bligh and Dyer.²⁰⁾ After the electrophoresis, protein was stained with Coomassie brilliant blue R-250 and dried. The density of each band was measured at 550 nm by a Shimadzu dual-wavelength flying-spot scanner CS-9000.

Measurement of Cholesterol Content The amount of total cholesterol (TC) and free cholesterol (FC) was determined enzymatically using Determiner TC and FC (Kyowa Medics Co.) respectively. The amount of CE was determined as the difference between the amount of TC and FC.

RESULTS

The effects of EGCG, α -tocopherol, myricetin, querce-

Fig. 1. Inhibitory Effects of Various Antioxidants on the Cu²⁺-Mediated Conjugated Diene Formation in LDL

LDL (100 μ g protein/ml in PBS) was incubated with 5 μ M CuSO₄ at 37 °C in the presence or absence of 0.5 μ M antioxidant. Conjugated diene formation was measured by determining the absorbance at 234 nm every 10 min for 5 h. Results are expressed as the absolute absorbance at 234 nm. —○—, control; —△—, α -tocopherol; —□—, BHT; —▲—, myricetin; —×—, theaflavin; ---×---, EGCG; —■—, quercetin.

TABLE I. Effects of Tocopherol Analogues on the Onset of Conjugate Diene Formation in LDL

	Lag-time (min)
None	15.0
α -Tocopherol	77.5
β -Tocopherol	52.5
γ -Tocopherol	52.5
δ -Tocopherol	45.0
BHT	105.0

LDL (100 μ g prot./ml) was incubated with 5 μ M CuSO₄ at 37 °C in the presence or absence of 1.0 μ M tocopherol analogues.

TABLE II. Effects of Sesaminol and Curcuminoid Derivatives on the Onset of Conjugate Diene Formation in LDL

	Lag-time (min)
None	10.9
Curcumin	60.0
Tetrahydrocurcumin	90.0
Sesaminol	510.0
BHT	45.0

LDL (100 μ g prot./ml) was incubated with 5 μ M CuSO₄ at 37 °C in the presence or absence of 0.5 μ M sesaminol and curcuminoid derivatives.

tin, and theaflavin on conjugated diene formation in LDL were examined by incubating LDL with 5 μ M CuSO₄. As shown in Fig. 1, the lag-time in the absence of antioxidant was 21.4 min. However, the lag-time was prolonged up to 235.7 min in the presence of 0.5 μ M antioxidant. The effects based on the time of prolongation were in the order: quercetin > EGCG > theaflavin $\geq$ myricetin > BHT > α -tocopherol.

Table I shows the effects of α -, β -, γ -, and δ -tocopherol on the lag-time for conjugated diene formation in the Cu²⁺-mediated oxidation of LDL. LDL was incubated with 5 μ M CuSO₄ in the presence of 1 μ M tocopherol analogues. The extent of lag-time prolongation by tocopherol analogues was in the order: α - > β - = γ - > δ - analogues. The antioxidative effects of tocopherols on conjugated diene formation in LDL were weaker than that of BHT, the reference antioxidant.

Fig. 2. Inhibition of the Cu^{2+} -Mediated CE Degradation in LDL by EGCG, BHT, and α -Tocopherol

LDL (200 μg protein/ml in PBS) was incubated with 5 μM CuSO_4 at 37°C in the presence or absence of antioxidant. After incubation, EDTA (final concentration 10 mM) was added to prevent any further oxidation and the amount of CE was determined as described in Materials and Methods. The results are represented as mean $\pm$ S.D. for triplicate determinations. Significance vs. control (with 5 μM CuSO_4) is represented by * ($p < 0.05$) and ** ($p < 0.01$).

Fig. 3. Inhibition of the Cu^{2+} -Mediated Apo B 100 Fragmentation in LDL by EGCG, BHT, and α -Tocopherol

LDL (200 μg protein/ml in PBS) was incubated with 5 μM CuSO_4 at 37°C in the absence (lane 2) or presence of 0.5 μM EGCG (lane 3), BHT (lane 4), or α -tocopherol (lane 5) for 1 h. After incubation, EDTA (final concentration 10 mM) was added to prevent any further oxidation. Approximately 12 μg protein of the delipidated LDL was applied to SDS-PAGE (3–15% gradient). After electrophoresis, each spot was stained with Coomassie brilliant blue R250. Lane 1 contained standard molecular weight makers, and lane 6 was native LDL.

The effects of lipid-soluble plant phenols, such as sesaminol and curcuminoids, on conjugated diene formation are shown in Table II. LDL was incubated with 5 μM CuSO_4 in the presence of 0.5 μM sesaminol and curcuminoids. Curcuminoids such as curcumin and tetrahydrocurcumin prolonged the lag-time slightly.

However, 0.5 μM sesaminol prolonged the lag-time by 510.0 min.

TC and CE decrease when LDL is incubated with CuSO_4 , as reported previously.¹⁵ Figure 2 shows the amount of CE when LDL was incubated with 5 μM CuSO_4 in the presence of EGCG or α -tocopherol for 8 h. The amount of CE decreased to 43.1% in the absence of antioxidants. This decrease, however, was significantly inhibited by 2.5 ($P = 0.0116$), 5.0 μM EGCG ($P = 0.0010$) or 5.0 μM BHT ($P = 0.0021$). The inhibitory effect of 5.0 μM EGCG was stronger than that of BHT ($P = 0.0098$). This effect was not observed in the presence of 2.5 or 5.0 μM α -tocopherol.

The inhibitory effect of EGCG or α -tocopherol on apo B 100 fragmentation was studied using SDS-PAGE (Fig. 3). The band of apo B 100 was not detected when LDL was incubated with CuSO_4 for 60 min. In the presence of 0.5 μM EGCG, the fragmentation of apo B 100 was inhibited and 74.2% of apo B 100 remained in native LDL. However, only 20% of apo B 100 remained in native LDL in the presence of 0.5 μM BHT. In addition, fragmentation was not inhibited by 0.5 and 1.0 μM α -tocopherol.

DISCUSSION

LDL can be oxidatively modified *in vitro* to be taken up by macrophages through scavenger receptors. This process is supposed to be a key step in the formation of cholesterol-laden macrophage foam cells in atherosclerotic lesions.¹

There are several reports suggesting that naturally occurring antioxidants in the diet may play a role in inhibiting the oxidative modification of LDL and hence they might act as antiatherosclerotic agents. de Whalley *et al.* reported¹⁶ that flavonoids such as quercetin strongly inhibited the modification of ^{125}I -labeled LDL by macrophages and prevented conversion of LDL to the modified form. The cell-mediated conversion of LDL to the form taken up by scavenger receptors was completely inhibited by the presence of 3 μM quercetin or 100 μM myricetin. They suggested that the flavonoids appeared to protect the macrophage-mediated LDL oxidation by protecting the endogenous α -tocopherol from being consumed. Many, if not all, aglycone flavonoids are rather lipophilic^{21,22} and they may penetrate into lipoproteins to protect LDL oxidation directly or indirectly through the contact with endogenous tocopherols. Mangiapane reported²³ that (+)-catechin inhibited the Cu^{2+} - and cell-mediated oxidation of LDL, and the LDL reisolated from the incubation mixture with 20 $\mu\text{g}/\text{ml}$ (about 70 μM) (+)-catechin was endocytosed and degraded at rates similar to that of native LDL. We have recently demonstrated that EGCG exerts strong antioxidative effects on the Cu^{2+} -mediated oxidative modification of LDL.¹⁵

In the present study, we have compared the antioxidative effects of various naturally occurring antioxidants with that of EGCG on the Cu^{2+} -mediated oxidative modification of LDL. We have used quercetin, myricetin, sesaminol, curcumin, tetrahydrocurcumin, theaflavin, and tocopherol analogues as natural antioxidants, which have

phenolic hydroxy groups in their chemical structures.

The inhibitory *in vitro* experiments on the oxidative impairment of LDL by these antioxidants were carried out using purified porcine serum LDL and incubating at 37°C in the presence of 5 μ M CuSO₄. The oxidation of LDL was monitored by the increase in absorbance due to conjugated diene formation. Results showed that sesaminol and quercetin inhibited the Cu²⁺-mediated LDL oxidation to a greater extent than EGCG. Myricetin, theaflavin, curcumin, tetrahydrocurcumin, and tocopherol analogues were less effective than EGCG.

In our previous report,¹⁵⁾ we showed that the antioxidative effect of (–)-epicatechin (EC), having no 5'-hydroxy group in its structure was stronger than that of (–)-epigallocatechin (EGC), which possess a 5'-hydroxy group. We found in the present study that the effect of quercetin having no 5'-hydroxy group, was also stronger than myricetin, having a 5'-hydroxy group in its structure. The presence of a 5'-hydroxy group in the structure of a compound, therefore, seem to weaken its antioxidative effect on LDL oxidation. The antioxidative activity of flavonol derivatives on LDL oxidation were stronger than that of flavan-3-ol derivatives in general. However, since the activity of EGCG, which belongs to the flavan-3-ol group, was stronger than that of myricetin, the role of the galloyl group might be important in inhibiting LDL oxidation. The antioxidative effect of theaflavin on LDL oxidation was slightly weaker than EGCG. Similar results were obtained in the *tert*-butylated hydroperoxide-induced peroxidation of rat liver where the IC₅₀ was 8.7 $\times 10^{-6}$ M for theaflavin and 5.6 $\times 10^{-6}$ M for EGCG, respectively.¹¹⁾ Sesaminol exhibited the strongest antioxidative activity in this experiment. Among the phenolic and β -diketone type antioxidants, tetrahydrocurcumin exhibited stronger activity than curcumin, as observed in a previous report.¹⁷⁾

The α -tocopherol present in LDL is known to play an important role in inhibiting the lipid peroxidation of LDL.⁸⁾ Exogenously added α -tocopherol, however, produced a weaker inhibition of lipid peroxidation than other natural antioxidants (Fig. 1). To protect the Cu²⁺-mediated LDL oxidation, it may be important to protect not only the direct but also the indirect oxidation of LDL which will be produced by endogenous antioxidant (tocopherols, β -carotene, lycopene *etc.*) consumption during oxidation.⁸⁾ The antioxidative and comparative effects of natural antioxidants as well as their effects on CE and apo B 100 fragmentation need to be carefully

evaluated after kinetic analysis of the disappearance of endogenous antioxidants in LDL; this is now in progress.

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