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Green tea polyphenols (flavan 3-ols) prevent oxidative modification of low density lipoproteins: An ex vivo study in humans

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Oxidation of low density lipoprotein (LDL) plays crucial roles in atherogenesis. We previously reported that green tea polyphenols (flavan 3-ols), especially epigallocatechingallate (EGCg) and epicatechingallate, exerted potent inhibitory effects on LDL oxidation in vitro. To examine whether intake of green tea polyphenols renders LDL resistant to ex vivo oxidation in humans, 22 male volunteers aged between 22 and 32 years were recruited and assigned the same dietary regimen for 2 weeks. After a 1-week baseline period, they were equally divided into two groups: control and tea. The tea group ingested 300 mg of green tea polyphenol extract twice daily for 1 week. Plasma EGCg concentration at the end of the experiment was 56 nmol/L on average (56% in free form) in the tea group; no EGCg was detected before the experiment. Plasma concentrations of lipids, ascorbate, α-tocopherol, and lipid peroxides did not change before and after the experiment in either group, but β-carotene was higher in the tea group ($P < 0.01$ by paired Student's t-test). LDL (0.1 mg/mL) was incubated with 5 μM Cu^{2+} and the oxidation was measured by absorbance at 234 nm. The lag time was significantly prolonged by 13.7 min in the tea group ($P < 0.05$ by paired Student's t-test, before versus after), whereas such a change was not observed in the control group. These results suggest that daily consumption of seven to eight cups (approximately 100 mL each cup) of green tea may increase resistance of LDL to in vivo oxidation, leading to reduction in the risk of cardiovascular diseases. (J. Nutr. Biochem. 11:216–222, 2000)

Keywords: green tea polyphenols; humans; ex vivo; LDL oxidation; lag time; vitamins

Introduction

In the subendothelial space, low density lipoprotein (LDL) is converted to oxidized forms through contact with macrophages and endothelial and smooth muscle cells. Oxidatively modified LDL expresses chemotactic and adhesion molecules on the surface of endothelial cells. Macrophages in the subendothelial cells intake modified LDL via scavenger receptors, leading to the formation of lipid-laden foam cells, the hallmark of early atherosclerotic lesions.[1,2] Thus, the prevention of LDL oxidation is assumed to be one of the important measures for preventing atherosclerosis.

Plasma contains natural occurring antioxidants such as ascorbic acid, α-tocopherols, carotenoids, and various types of flavonoids. Oxidative modification of LDL occurs when these antioxidants present in the plasma and inside LDL are largely consumed.[3] An epidemiologic study shows that European populations with higher plasma concentrations of natural antioxidants, ascorbic acid, and α-tocopherol have lower incidences of coronary heart disease.[4] There are also several epidemiologic investigations that show that flavonoid intake is inversely associated with mortality from coronary heart disease.[5–8] In addition, the Oppland County

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Study,[9] Scottish Heart Health Study,[10] and a study by Sesso et al.[11] indicated inverse correlation between black tea consumption and the risk of coronary heart diseases. However, the effect is still not conclusive.[12]

Green tea and black tea originate from the same plant, the *Camellia sinensis*. Green tea is produced by exposing the fresh shoot of the leaves to hot stream for 1 min to inactivate polyphenol oxidase, then drying. In contrast, black tea is produced by keeping the fresh shoot at room temperature (22–27°C) for 16 to 20 hr, a fermentation procedure, then cutting and drying. Both teas contain antioxidative polyphenols[13]; polyphenols in green tea consist of flavan 3-ols such as (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechingallate (ECg), and (−)-epigallocatechingallate (EGCg), whereas black tea contains theaflavins and thearubigins, complex condensation products produced during the "fermentation" process in addition to catechins.

We have already shown that these green tea polyphenols have a variety of pharmacologic effects: antioxidative,[14] antimutagenic,[15] anti-cancer promoting,[16] and hypolipidemic.[14] More recently, we also reported that these polyphenols exert potent inhibitory effects on Cu^{2+}-mediated oxidative modification of LDL.[17,18] Because green tea is the most commonly consumed beverage in Japan, we investigated whether intake of green tea polyphenols decreases the susceptibility of LDL to oxidative modification in humans.

Materials and methods

Subjects

Twenty-two male volunteers (12 smokers and 10 nonsmokers) aged between 22 and 32 years participated in this study. All subjects were willing to participate and gave informed consent. They were normolipidemic and did not take any medication, vitamin supplements, or special dietary additives. Among them, 12 had a smoking habit and the others were nonsmokers. They were equally allocated into two groups. The study protocol was approved by the ethics committee of our university.

Experimental design

During a 2-week period, volunteers were kept strictly under the same dietary regimen including refreshments. They were restricted to drinking green or black tea and fruit or vegetable juice. After a 1-week baseline period, they were equally divided into two groups (control and tea) in respect to body mass index and serum lipid concentration. The tea group ingested 300 mg of green tea polyphenol extract (Polyphenon E®) twice daily just before breakfast and dinner for 1 week.

Blood sampling

Blood (15 mL) was drawn from the vein in ethylenediamine tetraacetic acid (EDTA)-containing tubes before breakfast after an overnight fast. In the tea group blood was collected 1 hr after the intake of the green tea at the end of the experiment. Plasma was separated by low-speed centrifugation, immediately frozen in small portions in a methanol bath at −90°C, and stored at −80°C until used for LDL preparation and lipid assay. Plasma samples for ascorbate assay were deproteined with an equal volume of 10% (w/v) metaphosphoric acid; the supernatant was stored at −80°C. Plasma samples for catechins assay were mixed with 1/10 volume of ascorbate-EDTA solution [0.4 M NaH_2PO_4 buffer containing 20% (w/v) ascorbate and 0.1% (w/v) EDTA · 2Na, pH 3.6], frozen in a methanol bath at −90°C in small portions, and stored at −80°C.

Preparation and oxidation of LDL

LDL was separated mainly by the method of Chung et al.[19] Briefly, all plasma samples for LDL preparation were defrosted and the density was adjusted with KBr to 1.30 g/mL, layered under 10 mL saline containing 0.01% (w/v) EDTA · 2Na, and ultracentrifuged at 200,000 × g for 2.5 hr at 4°C in a vertical rotor (RPV-50T, Hitachi, Tokyo, Japan) using a ultracentrifuge (70P-72, Hitachi). After removing 11 mL of the top layer, the lower layer was fractionated into 0.3 mL each from the top using a density gradient fractionator (DGF-U, Hitachi). LDL fractions (no. 6–10) were pooled and the protein was adjusted to 0.1 mg/mL with phosphate-buffered saline (PBS) and dialyzed by desalizer II (Atto, Tokyo, Japan) against five changes of 900 mL of 10 mM PBS (pH 7.4) containing 1 μM EDTA for 36 hr at 4°C. LDL obtained by this method (0.1 mg protein/mL) was incubated for varying times in the presence of 5 μM $CuSO_4$ at 37°C, and absorbance at 234 nm was monitored for 3 hr at 20-min intervals using a spectrophotometer (U-2000, Hitachi).

Extraction of catechins from plasma and analysis by high performance liquid chromatography

Extraction of catechins from plasma and analysis by high performance liquid chromatography (HPLC) was carried out mainly by the method of Lee et al.[20] Plasma samples (220 μL containing ascorbate-EDTA · 2Na) were mixed vigorously with 200 μL of 0.1 M sodium acetate (pH 5.0, adjusted with 1 N HCl) and 500 μL ethyl acetate, and the mixture was centrifuged at 1,000 × g for 5 min at 4°C. The aqueous layer was again extracted twice with 500 μL of ethyl acetate, and all three supernatants were pooled. The supernatant containing free catechins was mixed with 10 μL of 1% (w/v) ascorbate and the solvent was dried under a stream of nitrogen gas. The pooled aqueous layer containing the conjugated forms was pooled and incubated for 3 hr with 500 units of β-glucuronidase and 40 units of sulfatase at 37°C, the formed free forms of catechin were extracted with ethyl acetate three times as descrived above. The residue was dissolved in 40 μL of the mobile phase solvent, and 10 μL of the sample solutions was injected into a HPLC system (Waters Millenium System, Waters, Milford, MA USA). The analytical condition was as follows: column: Capcell Pack C_{18} UG (4.6 × 250 mm), mobile phase: CH_3CN · ethylacetate · phosphate buffer ($NaH_2PO_4 \cdot 2H_2O$ 8 g, $Na_2HPO_4 \cdot 12H_2O$ 0.94 g, H_3PO_4 0.5 mL/900 mL H_2O, pH 4.9) = 12:0.6:90, flow rate: 0.8 mL/min, column temperature: 30°C, detection: PDA (Waters) ultraviolet 280

nm, and ECD (Bioanalytical System, Inc., West Lafayette, IN USA) 30°C, 70 mV.

Other measurements

Plasma cholesterol and triacylglycerols were enzymatically analyzed with Sterozyme 545 and Determiner LTG, respectively. The plasma ascorbate concentration was determined spectrophotometrically after derivatization with 2,4-dinitrophenylhydrazine.[21] α-Tocopherol and β-carotene were extracted from plasma and analyzed by HPLC according to methods by Ueda and Igarashi[22] and Stacewicz-Sapuntzakis et al.,[23] respectively. 2,2,5,7,8-Pentamethyl chlomanol was used as an internal standard. Thiobarbituric acid-reactive substances (TBARS) were measured according to the method of Yagi et al.[24] with some modification.[17] Protein in LDL was determined by the method of Lowry et al.[25]

Materials

α-Tocopherol was obtained from Eisai (Tokyo, Japan); L-ascorbic acid from Wako Pure Chemicals (Osaka, Japan); 1,1,3,3-tetraethoxypropane from Tokyo Kasei (Tokyo, Japan); β-carotene, β-glucuronidase (type X-A), and sulfatase (type VIII) from Sigma Chemical Co. (St. Louis, MO USA); Sterozyme-545 from Fuji Rebio (Tokyo, Japan); Determiner LTG from Kyowa Medics (Tokyo, Japan); green tea polyphenol extract (Polyphenon E) was supplied by Dr. Yukihiko Hara, Food Research Laboratories, Mitsui Nohrin Co. Ltd. (Tokyo, Japan).

Statistical analysis

Data are shown as mean ± SE for number of subjects in *Tables 2*, *3*, and *4*. Statistical significance between two groups was analyzed by Student's *t*-test in *Tables 2* and *4* and *Figure 1*. Additionally, data in *Table 4* and *Figure 1* were analyzed by paired Student's *t*-test (before versus after) within group. These analyses were performed with Stat View J5.0 (SAS Institute Inc., Cary, NC USA).

Results

Subjects in this study

Twenty-two male volunteers (average age 24.5 years) had meals and refreshments under the same dietary regimen. Green or black teas and fruit or vegetable juices were not allowed during the 2 weeks. After a baseline period, volunteers were divided into two groups: control and tea. The tea group had 300 mg of powdered green tea polyphenol extract Polyphenon E, a commercially available food supplement, twice daily before breakfast and dinner for 1 week. *Table 1* shows the composition of tea catechins in the extract used in this experiment. Intake of 600 mg of the tea extract roughly corresponded to drinking seven to eight cups (approximately 100 mL) of green tea.

Plasma catechin concentration

Table 2 shows plasma EGCg concentration before and after 1-week intake of green tea extract. Catechins in plasma were analyzed by HPLC. No catechins were detectable before the experiment, but among various catechins only EGCg, the principal component of green tea extract, was detected at the end of the experiment. A total 56 nmol/L EGCg was found in the tea group, with 52% in free form and 48% in conjugated forms. In the control group less than 1% of EGCg found in the tea group was detected in conjugated forms.

Table 1 Composition of tea extracts

Catechins	Contents (weight %)
(−)-Epigallocatechin-gallate	58.4
(−)-Epigallocatechin	11.7
(−)-Epicatechin	6.6
(+)-Gallocatechingallate	1.6
(−)-Epicatechingallate	0.5
Caffeine	0.4

Changes in plasma lipids, antioxidative vitamins, and TBARS

Table 3 shows the changes in plasma lipids, antioxidative vitamins, and TBARS. Plasma concentrations of lipids (cholesterol, triacylglycerols), ascorbate, α-tocopherol, β-carotene, and TBARS did not differ between each group before and after the experiment. In addition, there was no significant change in these concentrations between smokers and nonsmokers. *Figure 1* shows individual differences in these plasma concentrations before and after the experiment. β-Carotene concentration only was significantly elevated in the tea group, but the other concentration were unaltered in both control and tea groups, although α-tocopherol tended to increase in the tea group.

Effects of tea intake on Cu^{2+}-mediated LDL oxidation

LDL prepared from individual plasma was subjected to Cu^{2+}-mediated oxidation at 37°C, and the absorbance of

Table 2 Changes in plasma EGCg concentration by 1-week ingestion of tea extracts

	EGCg (nmol/L)*			
	Control		Tea group	
EGCg	Before†	After†	Before	After
Free	ND	ND	ND	29.2 ± 8.98[a]
Conjugated	ND	0.76 ± 0.62	ND	22.0 ± 18.0[a]
Total	ND	0.76 ± 0.62	ND	56.0 ± 14.4[a]

Men aged 24.5 years on average were assigned the same dietary regimen for 2 weeks. The tea group was given tea polyphenol extract, 300 mg × 2/day before the breakfast and dinner for 1 week. Blood samples were drawn after an overnight fast before the breakfast, and 60 min after intake of the tea extract in the tea group at the end of experiment.
*Data represent mean ± SE ($n = 7$).
†Before and after the experimental period.
[a]$P < 0.001$ by Student's *t*-test for tea group versus control (after).
EGCg–(−)-epigallocatechin-gallate. ND–not detected.

Table 3 Changes in plasma lipids, antioxidative vitamins, and TBARS after 1-week ingestion of tea extract

Group	Control	Tea
Age (y)	24.1 ± 0.4	24.9 ± 0.9
Body weight (kg)	63.6 ± 2.7	63.3 ± 2.4
BMI (kg/m^2)	21.2 ± 0.6	21.5 ± 0.5
Plasma total cholesterol (mmol/L)		
0 week	4.90 ± 0.26	4.87 ± 0.30
1 week	4.37 ± 0.28	4.44 ± 0.24
2 weeks	5.01 ± 0.27	5.00 ± 0.21
Plasma triacylglycerols (mmol/L)		
0 week	0.92 ± 0.10	1.38 ± 0.34
1 week	1.13 ± 0.10	1.25 ± 0.17
2 weeks	1.26 ± 0.16	1.28 ± 0.15
Antioxidative vitamins		
Ascorbate (μmol/L)		
0 week	28.2 ± 3.6	29.2 ± 2.0
1 week	29.1 ± 11.3	29.6 ± 9.3
2 weeks	27.3 ± 11.9	26.2 ± 9.5
α-Tocopherol (μmol/L)		
0 week	14.1 ± 0.9	13.4 ± 0.8
1 week	13.8 ± 1.1	14.1 ± 1.7
2 weeks	13.9 ± 1.2	15.6 ± 2.0
β-Carotene (μmol/L)		
0 week	0.50 ± 0.22	0.43 ± 0.15
1 week	0.63 ± 0.15	0.41 ± 0.06
2 weeks	0.60 ± 0.17	0.75 ± 0.09
TBARS in plasma (μmol MDA/L)		
0 week	4.50 ± 0.88	4.76 ± 1.14
1 week	4.08 ± 0.38	4.11 ± 0.38
2 weeks	4.57 ± 0.42	4.41 ± 1.40

See *Table 2* for Experimental details.
Data represent mean ± SE (n = 11).
TBARS–thiobarbituric acid reactive substances. BMI–body mass index. MDA–malondialdehyde.

234 nm was monitored for conjugate diene formation. *Table 4* shows lag time and propagation rate of LDL oxidation. To compare the lag time before and after the experiment as accurately as possible, all LDL samples were prepared and oxidized by Cu^{2+} in a single run experiment. The lag time in the tea group was significantly prolonged by 13.7 min after the experiment compared with that before the experiment (by paired Student's *t*-test). In contrast, there was no significant change in lag time before and after the experiment in the control group. The propagation rate did not differ before and after the experiment in either group. Again, no difference in lag time and propagation rate was observed between smokers and nonsmokers.

Discussion

When 10 g of green tea leaves were soaked in 430 mL hot water (90°C) for 1 min, approximately 280 mg tea catechins were extracted in the resulting tea beverage. This is a standard method to prepare Japanese green tea. Therefore, intake of 300 mg powdered green tea polyphenol extract (Polyphenon E) containing approximately 240 mg catechin, twice a day, in our experiment is almost equivalent to the consumption of 740 mL (7–8 cups) of tea.

LDL contains various endogenous antioxidants, including α- and γ-tocopherol, β-carotene, lycopene, and retinyl stearate. While the oxidative modification of LDL occurs, these antioxidants are assumed to be consumed first. Following the depletion of its endogenous lipophilic antioxidants, lipid peroxidation can enter into a propagating chain reaction and the formation of various aldehydes is observed. However, when water-soluble antioxidants such as urate and ascorbate are present outside LDL particles, these antioxidants could retard the destruction of the endogenous antioxidants in LDL by reductive recycling.[3] Green tea intake did not change plasma ascorbate concentration, but tended to increase α-tocopherol concentration and significantly increased the β-carotene concentration. These results suggest that the presence of tea polyphenols with lipid and water-soluble (dual) properties in plasma might prevent the in vivo consumption of the lipid soluble antioxidants such as β-carotene at least.

The lag time of LDL oxidation in the tea group was significantly prolonged by 14 min compared with the lag time before the experiment ($P < 0.05$ by paired Student's *t*-test). In contrast, the lag time in the control group was unchanged before and after the experiment. In an intervention study of α-tocopherol,[26,27] an approximately 15-min prolongation of lag time was observed in Cu^{2+}-mediated oxidation of LDL from the group whose plasma α-tocopherol concentration was 1.5 to 1.8 times higher than that of the baseline concentration. For this elevation of α-tocopherol in the plasma, 200 to 400 mg daily supplementation of α-tocopherol seems to be needed. Almost the same effect was attained by the daily intake of 480 mg catechin in the present experiment.

Princen et al.,[28] Reaven et al.,[29] and Dugas et al.[30] reported that β-carotene supplementation did not protect LDL oxidation in humans, whereas Lin et al.,[31] Steinberg and Chait,[32] and Dugas et al.[33] reported that β-carotene at low doses showed a protective effect on LDL oxidation. Dugas et al.[33] recently reported that three- to sixfold enrichment of LDL with β-carotene through dietary supplement was more effective than 11- to 12-fold enrichment achieved by an in vitro method. Therefore, the elevation of plasma β-carotene (a scavenger of singlet oxygen[34]) together with a slight increase in plasma α-tocopherol by intake of the green tea extract may contribute at least partly to the reduction of the susceptibility of LDL to oxidation.

Consumption of red wine, soybean phytoestrogens,[35] and polyphenols found in cocoa[36] and licorice extract[37] are reportedly shown to increase the resistance of LDL to ex vivo oxidation. There is also evidence against it.[38] Several epidemiologic investigations[9–11] indicate inverse correlation between black tea consumption and the risk of coronary heart disease. However, studies examining the effect of tea consumption on LDL oxidation ex vivo are inconsistent. Serafini et al.[39] examined in vivo antioxidant effect of green and black teas. They reported that antioxidant activity of plasma taken 30 to 80 min after male volunteers ingested 300 mL of green or black tea was higher than that of the control plasma. Van het Hof et al.[40] tested the efficacy of 4 weeks of green or black tea consumption on ex vivo LDL oxidation in 18- to 65-year-old healthy men and women. The tea group had six cups (900 mL) of tea for 4 weeks, but

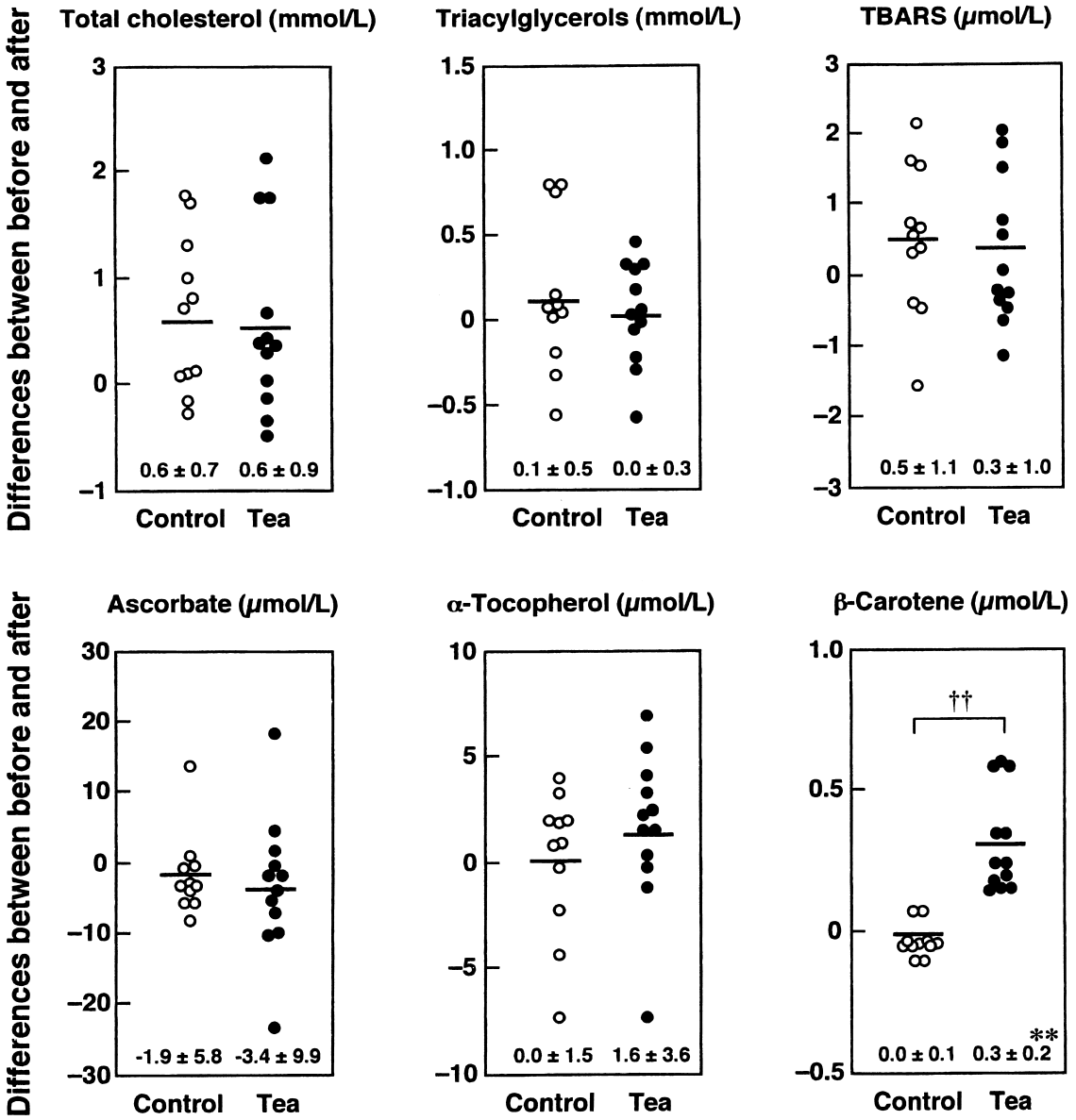

Figure 1 Individual differences in plasma lipids, thiobarbituric acid reactive substances (TBARS), and antioxidative vitamins before and after the experimental period. $^{**}P < 0.01$ by paired Student's *t*-test for before versus after; $^{††}P < 0.01$ by Student's *t*-test, control versus tea group. Data indicate mean ± SE ($n = 11$).

resistance of LDL for copper-induced oxidation was not changed by green or black tea ingestion, although green tea slightly increased total antioxidant activity. Princen et al.[41] conducted a similar examination in smokers. They too failed to show antioxidative activity of tea in ex vivo LDL oxidation. Only one positive effect of tea consumption on LDL oxidation has been reported by Ishikawa et al.[42] Healthy male volunteers (N = 22) aged 22 ± 1 years were recruited and half of them were given five cups (750 mL) of black tea (11 g) per day for 4 weeks. They found that consumption of black tea significantly prolonged the lag time of LDL oxidation by Cu^{2+}. The subjects lived in a boarding house and ate the same variety of foods (personal communication with Dr. T. Ishikawa).

The investigation performed by Ishikawa et al.[42] and our present study indicate that ingestion of black or green tea prevented oxidation of LDL ex vivo, whereas van het Hof et al.[40] and Princen et al.[41] did not find the effect of tea ingestion on LDL oxidation. Discrepancy of the results might be derived from differences in the experimental procedures. In the investigation that reported positive results, the subjects consisted of men only within a small age range (22 ± 1 or 24 ± 1 years), all subjects were on the same dietary regimen, and the data of lag time of LDL oxidation were analyzed by paired Student's *t*-test. In contrast, in the investigations that reported negative results,[40,41] the ages of the subjects are of wide age range (18–65 years, average age: 38 ± 12 and 34 ± 12) and both

Table 4 Effect of 1-week ingestion of tea on Cu^{2+}-mediated LDL oxidation

Group	Control	Tea	Significance versus control
Lag-time (min)			
Before*	68.1 ± 2.9	64.6 ± 2.4	
After*	68.3 ± 2.0	79.6 ± 5.9	
Individual differences	0.2 ± 2.8	13.7 ± 6.1[a]	$P < 0.05$[b]
Propagation rate (min/mg/LDL protein)			
Before	2.31 ± 0.16	2.15 ± 0.15	
After	2.27 ± 0.21	2.21 ± 0.21	NS

Low density lipoprotein (LDL) was prepared by discontinuous ultracentrifugation and dialyzed. LDL (0.1 mg protein/mL) was incubated in the presence of 5 μM $CuSO_4$ at 37°C and the change in the absorbance at 234 nm was monitored.
Data represent mean ± SE (n = 11).
*Before and after the experimental period.
[a]$P < 0.05$ by paired Student's t-test for before versus after within group.
[b]$P < 0.05$ by Student's t-test for before versus after between groups.

male and female subjects were included. They ate their own food including some limited amounts of fruits and fruit juice. Moreover, data were not analyzed by paired Student's *t*-test. To eliminate genetic factors, various environmental factors, and factors related to sex and age, it would be necessary to analyze data by paired Student's *t*-test.

In our previous in vitro experiment with porcine LDL, the presence of 0.5 μM catechins markedly prevented Cu^{2+}-mediated oxidation.[17,18] Lag times (minutes) were 15 (without antioxidants), 45 (butylated hydroxy toluene), 100 (EGC), 123 (C), 143 (EC), 161 (ECg), and 188 (EGCg). In our present experiment plasma EGCg concentration in the tea group was 56 nM at the end of the study. To examine the efficacy of catechins at the lower concentrations, human LDL was incubated with 5 μM Cu^{2+} in the presence of varying concentrations of EGCg or Trolox, a reference compound. The lag times (minutes) were as follows: 51 (control), 60 (0.1 μmol of EGCg/L), 67 (0.3 μmol of EGCg/L), 89 (0.5 μmol of EGCg/L), and 70 (0.5 μmol of Trolox/L; data not shown). When EGCg was incubated with plasma or bile, most of them disappeared in 30 min, whereas approximately 20% of the antioxidative activity still remained. Two new dimerized products of EGCg (P-1 and P-2) were isolated from the incubation mixture, and their antioxidative activities were comparable to those of EGCg.[43] Taken together with a significant increase (1.8-fold) in plasma β-carotene by tea intake and comparable antioxidant properties of EGCg metabolites to EGCg, ingestion of green tea would play protective roles against in vivo oxidation of LDL even at rather lower concentration of plasma EGCG itself. We also found that in our other experiment, long-term administration of green tea polyphenol extract inhibited progression of atherosclerosis in apo E-deficient mice.[44]

All these findings strongly suggest that daily consumption of green tea renders LDL resistant to in vivo oxidative modification either directly or via their metabolites, and indirectly by reducing the depletion of antioxidative vitamins, leading to the reduction in the risk of cardiovascular diseases.

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