Tea Catechins Prevent the Development of Atherosclerosis in Apoprotein E–Deficient Mice

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ABSTRACT Green tea contains various antioxidative flavan-3-ols (tea catechins), such as (–)-epigallocatechin gallate (EGCg, the major catechin), which exert potent inhibitory effects on LDL oxidation in vitro and ex vivo in humans. In this study, the antiatherogenic effects of tea catechins were examined in atherosclerosis-susceptible C57BL/6J, apoprotein (apo)E-deficient mice. Male apoE-deficient mice (10 wk old) were fed an atherogenic diet for 14 wk; during that time, one group (tea) was supplied drinking water supplemented with green tea extract (0.8 g/L), and another group (control) was offered the vehicle only. The tea extract consisted of the following (g/100 g): EGCg, 58.4; (–)-epigallocatechin (EGC), 11.7; (–)-epicatechin (EC), 6.6; (–)-gallolecchingallate (GCG), 1.6; (–)-epicatechin gallate (ECg), 0.5; and caffeine, 0.4. The estimated actual intake of tea catechin was 1.7 mg/d (d–mouse). Tea ingestion did not influence plasma cholesterol or triglyceride concentrations. Plasma lipid peroxides were reduced in the tea group at wk 8, suggesting that the in vivo oxidative state is improved by tea ingestion. Atheromatous areas in the aorta from the arch to the femoral bifurcation and aortic weights were both significantly attenuated by 23% in the tea group compared with the control group. Aortic cholesterol and triglyceride contents were 27 and 50% lower, respectively, in the tea group than in the control group. These results suggest that chronic ingestion of tea extract prevents the development of atherosclerosis without changing the plasma lipid level in apoE-deficient mice, probably through the potent antioxidative activity of the tea.

KEY WORDS: apoprotein E–deficient mice, atherosclerosis, catechins, antioxidants, aortic lipids

Oxidation of LDL plays a crucial role in the initiation mechanism of atherosclerosis (Berliner and Heinecke 1996, Wittram and Steinberg 1991). An epidemiologic study indicated that European populations with higher plasma concentrations of natural antioxidants, ascorbic acid and α-tocopherol have a lower incidence of coronary heart disease (Gey et al. 1987). Several epidemiologic investigations indicated that flavonoid intake is inversely associated with the mortality of coronary heart disease (Hertog et al. 1993 and 1997, Knekt et al. 1996, Rimm et al. 1996). In addition, the Oppland County Study (Stensvold et al. 1992), Scottish Heart Health Study (Brown et al. 1993) and a study by Sesso et al. (1999) all indicated an inverse correlation between black tea consumption and the risk of coronary heart disease. There have been no epidemiologic investigations, however, of green tea and coronary heart disease.

Green tea leaves (Camellia sinensis) contain antioxidative tea catechins consisting of various flavan-3-ols as follows: (+)-catechin (C), (–)-epicatechin (EC), (–)-epicatechin gallate (ECg), (–)-epigallocatechin (EGC) and (–)-epigallocatechin gallate (EGCg). Among them, EGCg with a hydroxy group at R1 and a galloloyl group at R2 is the principal component (Fig. 1). In contrast, black tea contains mainly thearubigins and theaflavins, complex condensation products of tea catechins. Tea catechins have a variety of pharmacologic effects, i.e., antioxidative (Lin et al. 1996, Yoshino et al. 1994), antimitogenic (Jain et al. 1989), anticarcinogenic (Katiyar et al. 1993, Wang et al. 1995, Yang et al. 1997), anticancer promoting (Nakamura et al. 1997), anti-inflammatory (Sano et al. 1999), antimicrobial (Yoshino et al. 1996) and hypolipidemic effects (Yoshino et al. 1994). Recently, Cao and Cao (1999) reported that EGCg inhibits angiogenesis.

We reported previously that green tea catechins exert po-
tent inhibitory effects on Cu$^{2+}$-mediated oxidative modification of LDL in vitro (Miura et al. 1994 and 1995). We also reported that daily consumption of tea catechins [equivalent to 740 mL (7–8 cups) of tea] for 1 wk significantly prolonged the lag time of LDL oxidation in humans (Miura et al. 2000). The present study investigated whether daily consumption of catechins prevents the development of atherosclerosis using atherosclerosis-susceptible C57BL/6J, apoE-deficient mice (apoE (−) mice), which were generated from Jackson Laboratories (Bar Harbor, ME) and bred in our laboratory. The Committee of the University of Shizuoka approved the protocol for experimental animals. Male apoE−/− (apoE (−) mice (n = 17) and wild-type apoE (+) mice (n = 16)) were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in our laboratory. The Committee of the University of Shizuoka approved the protocol for experimental animals. Male apoE−/− mice, 10 wk old, were divided into two groups (tea and control) and fed an atherogenic diet (CRF-1) containing 1.25 g/100 g cholesterol, 0.5 g/100 g sodium cholate, 12.5 g/100 g cocoa butter in CRF-1 (Oriental Yeast, Tokyo, Japan) for 14 wk (Table 1). When the atherogenic diet was given to apoE−/− mice starting at ages younger than 10 wk, growth was significantly impaired (data are not shown). Thus, the experiment was started when the mice were 10 wk of age. When mice were killed 10 and 12 wk after the start of the atherogenic diet, fatty plaques in the aorta were not evident. Therefore, in this study, animals were killed at 14 wk.

The tea extract (Polyphenon E) was given to the tea group via drinking water supplemented with tea extract (0.8 g/L) in 30 g/L sucrose during the 14-wk experimental period. The control group consumed 30 g/L sucrose. Sucrose was used to mask the bitterness of the tea extract. Drinking water containing the tea extract was freshly prepared and replaced every evening. Fluid intake was measured daily, and the amount of food ingested was measured once a week in the tea group. The average fluid intake on the previous day and the average amount of the diet ingested in the previous week were given to the control group during the experimental period. Blood samples were drawn from the retroorbital plexus every 4 wk, and from the abdominal aorta at the end of the experiment from mice lightly anesthetized with ether. Na$_2$EDTA (1 g/L) was added to the blood. Plasma was obtained by centrifuging the blood at 100 × g for 4°C and kept at −80°C until use.

**Fast protein liquid chromatography (FPLC) of lipoproteins.** Plasma lipoproteins were analyzed using an FPLC system (Pharmacia, Uppsala, Sweden). Filtered plasma (5 mL) was applied to a Superose 6 column (1 × 30 cm), eluted at a 0.5 mL/min flow rate with 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 0.1 mM Na$_2$EDTA and 0.2% NaN$_3$, and fractionated into 1-mL fractions. Total cholesterol in each fraction was measured enzymatically using Sterozyme 545.

**Plasma lipid peroxides.** To assess the in vivo oxidative state of lipoproteins, plasma lipid peroxides were measured through formation of thiobarbituric acid reactive substances (TBARS). The effects of various tea catechins on Cu$^{2+}$-mediated LDL oxidation were examined previously (Miura et al. 1994). Lipid hydroperoxides measured by a reaction with diphenyl-1-pyrenylphosphine using linoleic acid 13-hydroperoxide (Asaka et al. 1987) as a standard produced a result comparable to that measured by TBARS formation. Therefore, TBARS formation was examined in the present experiment. Measurement of TBARS was performed according to Yagi (1976) with slight modification. Fluorescent TBARS were extracted with butanol and the intensity was measured at 515 nm (excitation) and 535 nm (emission) using a Hitachi spectrofluorometer (F3010, Hitachi, Tokyo). TBARS were expressed as malondialdehyde (MDA; mol/mol) formed from 1,1,3,3-tetraethoxypropane, which was used as a reference compound.

**Analysis of atherosclerotic area in the aorta.** Each aorta from the arch to the femoral bifurcation was cleaned free of connective tissue and fat, and weighed after being lightly dried on filter paper. The aorta was cut longitudinally with scissors and pinned to a silicon plate to be photographed. The surface area of the aorta covered by lesions and the entire surface area were analyzed using NIH image software; the extent of atherosclerosis was expressed as a percentage of the entire surface area. Fatty streak lesion in aortic sinus was stained with Masson trichrome.

**Analysis of aortic lipids.** Immediately after measurement of the atherosclerotic area, each aorta was cut into small pieces and homogenized in 5 mL CHCl$_3$/methanol (2:1) mixture (Chiba et al. 1997). After soaking for 15 h at 4°C, the diced aorta was removed and the solvent was mixed vigorously with 2 mL of 5 g/L NaCl and

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**TABLE 1**

Composition of the atherogenic diet

<table>
<thead>
<tr>
<th>Component</th>
<th>g/100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRF-1</td>
<td>85.75</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.25</td>
</tr>
<tr>
<td>Sodium cholate</td>
<td>0.5</td>
</tr>
<tr>
<td>Cocoa butter</td>
<td>12.5</td>
</tr>
</tbody>
</table>

1 CRF-1: protein, 22.6 g; fat, 5.6 g; carbohydrate, 53.8 g; fiber, 3.3 g; ash, 6.6 g; water, 8.1 g; vitamins A, 3,783 IU; cholecalciferol, 503 IU; E, 21.2 mg; K, 0.16 mg; thamine, 4.44 mg; riboflavin, 3.06 mg; C, 14 mg; B-6, 1.26 mg; B-12, 12.2 µg; inositol, 431 mg; biotin, 27.8 µg; pantothenic acid, 7.07 mg; niacin, 14.6 mg; choline, 0.31 g; folic acid, 0.25 mg).

2 Cocoa butter: fatty acid composition g/100 g: 14:0, 0.1; 16:0, 25.8; 16:1, 0.3; 18:0, 34.5; 18:1, 35.5; 18:2, 2.9; 20:0, 11.1.
TEA CATECHINS PREVENT ATHEROSCLEROSIS

RESULTS

Composition and stability of tea extract. The green tea extract (Polyphenon E) contained EGCg (58.4 g/100 g) as the principal component (Table 2). Because tea catechins and EGCg, in particular, are easily oxidized, their stability during the feeding period was examined (Table 3). The catechins kept in brown drinking bottles remained ~90% intact after 24 h. When the solution was offered to the mice through the bottles, however, EGCg, EGC and ECg were reduced to 33, 37 and 67%, respectively. The air bubbles that entered into the bottles, however, EGCg, EGC and ECg were reduced to 33, 37 and 67%, respectively. The air bubbles that entered into the solution each time the mice drank appeared to have oxidized the catechins.

Body weights, lipoprotein profile, and time course of plasma cholesterol. It was confirmed by a preliminary experiment that the concentration of tea catechins present in the drinking water does not impair growth of apoE (+) mice. Sucrose was used to mask the bitter taste of the tea extract. The tea group consumed ~4 mL water/d; thus, mice ingested 3.2 mg of the tea extract per day. The calculated amounts of catechins remaining in drinking bottles after 12 h were EGCg, 66.2%; EGC, 68.4%; EC, 95.0%; and ECg, 83.6% (Table 3). Provided that mice consumed fluid containing catechins at this concentration on average throughout the day, and taking into account the composition in the tea extract (Table 2), the amount of the tea extract ingested would be ~1.7 mg (d · mouse).

Body weights at the end of the 14-wk experiment were significantly higher in the tea group than in the control group although they were pair-fed (Table 4). Figure 2 shows the lipoprotein profile of the apoE (+)-mice (A) and apoE (−)-mice (B). In contrast to the apoE (+)-mice (Fig. 2A), only VLDL were detected in apoE (−)-mice (Fig. 2B), and the levels of VLDL cholesterol increased with time of cholesterol feeding. Tea ingestion did not affect plasma total cholesterol concentration through the experimental period (not significant, two-way ANOVA) (Fig. 3) or plasma triglyceride concentrations (Table 4).

Plasma lipid peroxides. At the start of the experiment, the plasma TBARS concentrations were 6–8 μmol MDA/L without any difference between the two groups. By wk 8, the level had increased and was significantly lower in the tea group than in the control group (20.0 ± 4.90 (n = 7) vs. 30.3 ± 4.17 (n = 7) μmol MDA/L, P < 0.001).

Antiatherogenic effects of the tea extract. The atheroma-tous area, aortic weights, and aortic lipids in the tea and control groups are shown in Table 5. In the control group, 36% of the entire aortic area was covered with fatty plaque, whereas only 27% of the aortic area was covered in the tea group. Tea ingestion significantly attenuated the atherosclerotic area by 23% (P < 0.01). The atherosclerotic lesion area of the arch was similar in extent in both groups. In addition, there was no difference between the two groups in the degree of the lesion in aortic sinus stained with Masson trichrome at this advanced stage.

The weight of the cleaned aortas from the tea group was 23% lower than in the control group (P < 0.01), due to fewer fatty plaques and less smooth muscle proliferation. The cholesterol content in the tea group was 27% lower than in the control group (P < 0.01). Reduction of the triglyceride content was more marked than that for cholesterol; there was a 50% reduction in the tea group compared with the control group.

TABLE 2
Composition of tea extract1

<table>
<thead>
<tr>
<th>Catechins</th>
<th>mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-Epigallocatechin-gallate (EGCg)</td>
<td>584</td>
</tr>
<tr>
<td>(-)-Epigallocatechin (EGC)</td>
<td>117</td>
</tr>
<tr>
<td>(-)-Epicatechin (EC)</td>
<td>66</td>
</tr>
<tr>
<td>(+)-Gallocatechin-gallate (GCg)</td>
<td>16</td>
</tr>
<tr>
<td>(-)-Epicatechingallate (ECg)</td>
<td>5</td>
</tr>
<tr>
<td>Caffeine</td>
<td>4</td>
</tr>
</tbody>
</table>

1 Means of triplicate analyses.

TABLE 3
Stability of tea polyphenols in solution offered to mice1,2

<table>
<thead>
<tr>
<th></th>
<th>% remaining at 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1) Reserved in bottles</td>
</tr>
<tr>
<td>EGCg3</td>
<td>86.5</td>
</tr>
<tr>
<td>EGC</td>
<td>95.2</td>
</tr>
<tr>
<td>EC</td>
<td>89.9</td>
</tr>
<tr>
<td>ECg</td>
<td>91.8</td>
</tr>
</tbody>
</table>

1 Solution of (1) tea extract (0.8 g/L in 30 g/L sucrose solution) was kept for 24 h in brown drinking bottles at room temperature (25 ± 1°C).
2 Supplied to mice from the same brown bottles.
3 See Table 2 for catechin abbreviations.

TABLE 4
Body weights, plasma cholesterol and triglycerides, and oxidative state of plasma lipoproteins in apoprotein (apo)E-deficient mice fed an atherogenic diet with and without supplemental tea extract1

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>Tea group</th>
</tr>
</thead>
<tbody>
<tr>
<td>wk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body, g</td>
<td>0 ± 0.5</td>
<td>25.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>14 ± 0.7</td>
<td>26.5 ± 0.4**</td>
</tr>
<tr>
<td>Plasma cholesterol, mmol/L</td>
<td>14 ± 2.97</td>
<td>14.6 ± 3.14</td>
</tr>
<tr>
<td></td>
<td>83.2 ± 16.9</td>
<td>75.0 ± 20.1</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/L</td>
<td>0.89 ± 0.50</td>
<td>0.86 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>0.49 ± 0.20</td>
<td>0.85 ± 0.73</td>
</tr>
<tr>
<td>Plasma lipid peroxides,2 μmol MDA/L</td>
<td>6.48 ± 2.31</td>
<td>6.83 ± 1.09</td>
</tr>
<tr>
<td></td>
<td>30.3 ± 4.17</td>
<td>20.0 ± 4.90*</td>
</tr>
</tbody>
</table>

1 Values are means ± sd, n = 16 (control) or 17 (tea).
2 n = 7; MDA, malondialdehyde.
* P < 0.01 and ** P < 0.001: significantly different from control by Welch’s t-test and Student’s t-test, respectively.

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DISCUSSION

Tea catechins have potent inhibitory effects on LDL oxidation, in vitro (Miura et al. 1994 and 1995) and ex vivo (Miura et al. 2000). Thus, we tested the antiatherogenic effects of green tea extract in apoE-deficient mice in this study. Apoprotein E is involved in the clearance of chylomicrons and VLDL. Lack of apoE, therefore, causes accumulation of cholesterol-rich remnants in plasma, whose prolonged circulation is atherogenic.

Zhang et al. (1992) generated apoE-deficient mice by gene targeting. These mice develop atherosclerosis spontaneously and cholesterol loading accelerates atherosclerosis. Zhang et al. (1992) generated apoE-deficient mice by gene targeting. These mice develop atherosclerosis spontaneously and cholesterol loading accelerates atherosclerosis.

A previous in vitro experiment indicated that the components of tea catechins possess varying inhibitory effects on Cu2+ -mediated oxidation of LDL in the following order: EGCg > ECg > EC > C > EGC. The effect of t-BHT was ~25% that of EGCg and 50% that of EGC (Miura et al. 1994). EGCg and ECg also inhibited platelet aggregation 4 to 5 times more than aspirin (data not shown). To test ex vivo antioxidative effects of tea catechins in humans, we used tea extract whose components were analyzed precisely, rather than the pure component, because tea extract is commercially available in health food stores, and it is illegal to administer the pure components of tea catechin to humans. This study used a tea extract containing 58% of the most effective component, EGCg. Although the same amounts of food and fluid as consumed by the tea group were pair-fed to the control group, the body weights in the tea group were greater than those in control group. The reason remains unclear. Tea ingestion may relieve physiologic damage due to loading of the atherogenic diet because the diet greatly impaired growth in mice at ages younger than 10 wk.

Green tea is the most popular beverage in Japan and the principal component, EGCg, exerts a much stronger antioxidative effect than teaflavin and teaubidin, the major components of black tea on the basis of weight (Wiseman et al. 1997). Considering the catechin degradation in the drinking water supplied, mice ingested a daily average of ~1.7 mg tea catechin/mouse when they consumed 4 mL of the solution containing 0.8 g tea extract/L.

A standard way of preparing Japanese green tea is to soak 10 g of green tea leaves in 430 mL hot water (90°C) for 1 min. The resulting tea beverage contains ~280 mg tea catechins.

| TABLE 5
| Atheromatous area, aortic weights and aortic lipid contents in apoprotein apoE-deficient mice fed an atherogenic diet with and without supplemental tea extract for 14 wk1 |
| --- | --- | --- |
| Control | Tea group |
| Atheromatous area, %2 | 36.1 ± 8.25 | 27.7 ± 8.59a |
| Aorta, mg | 14.3 ± 2.8 | 11.0 ± 1.6c |
| Cholesterol, μmol/aorta | 0.830 ± 0.264 | 0.610 ± 0.189a |
| Triglycerides, μmol/aorta | 0.137 ± 0.077 | 0.068 ± 0.032b |

1 Values are mean ± SD, n = 16 (control) or 17 (tea).
2 % of whole aortic arch.
3 Student’s t test and Welch’s t test.
Japanese people daily ingest 413 mg tea catechins on average because they ordinarily drink 7 to 8 cups (740 mL) of green tea per day. On the basis of body weight (average Japanese, 60 kg and mice, 25 g), the intake of 1.7 mg tea catechin extract by mice per day corresponds to a human ingesting approximately eightfold the ordinary intake.

Daily ingestion of tea catechins for 14 wk prevented the accumulation of aortic cholesterol and triglycerides and atheroma formation in the aorta in apoe-deficient mice fed an atherogenic diet, without affecting plasma lipid concentration in comparison with the control group. This suggests that tea catechins rendered plasma lipoproteins of apoe-deficient mice less atherogenic.

A variety of plasma lipid-lowering agents exert antiatherogenic effects, but only a few agents have such effects without lowering plasma lipids. Among them are probucol, which possesses a strong antioxidant property (Kita et al. 1987), a lipoprotein lipase-enhancing agent, 4-dietroxyphosphonylmethyl N-[4-(3-methoxyphenyl)-5,7-dihydroxy-3-oxo-2-phenylpropan-2-yl]amine (Haenen et al. 1993), and a lipoprotein lipase-enhancing agent, 4-diethoxyphosphonylmethyl N-[3-methoxyphenyl] vinylamine (Hollman et al. 1997).

Hayek et al. (1997) fed red wine or its polyphenols, quercetin and catechin, to apoe-deficient mice fed a regular diet for 6 wk. They observed reduced susceptibility to LDL oxidation and an attenuation of the development of atherosclerotic lesions in the aortic arch in mice fed red wine or quercetin and, to a lesser extent, in mice fed catechins. It is difficult to compare their catechin results with the present study because the quality of the catechin they used was not described.

The mechanisms underlying the antiatherogenic effects of tea catechins are not clearly understood. Recent evidence from various lines of investigation, however, provides plausible explanations. Reactive oxygen species are involved in atherogenesis from various lines of investigation, however, provides plausible explanations. Reactive oxygen species are involved in atherogenesis (Schreck et al. 1991). The determination of lipid hydroperoxides with diphenyl-1-pyrenophosphine. Anal. Lett. 20: 797–807.


In conclusion, the present study, together with previous findings, suggests that chronic ingestion of green tea extract prevents the development of atherosclerosis without changing the plasma lipid levels, probably through the potent antioxidant activity.

LITERATURE CITED


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