Interaction of Tea Catechins with Lipid Bilayers Investigated with Liposome Systems

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Interaction of tea catechins with lipid bilayers was investigated with liposome systems, which enabled us to separate liposomes from the external medium by centrifugation. We found that epicatechin gallate had the highest affinity for lipid bilayers, followed by epigallocatechin gallate, epicatechin, and epigallocatechin. Epicatechin gallate and epigallocatechin gallate in the surface of lipid bilayer perturbed the membrane structure.

Key words: catechins; tea; EGCg; liposome; lipid bilayer

The biological activities of polyphenols have been examined by various methods in vitro and in vivo for prediction of their ability to prevent diseases. Epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECg), and epigallocatechin gallate (EGCg) referred to as tea catechins, are the major components of polyphenols in green tea infusions (Fig. 1). In recent in vitro studies, the effects of tea catechins on various assay items have been investigated to clarify the structure-activity relationship.1,4 In all cases, the activities of EC and EGC were lower than those of their corresponding gallic acid esters, i.e., ECg and EGCg. The reason why the gallic acid esters show high activity has not been identified.

We developed a method to measure the amount of polyphenols incorporated into the lipid bilayers of liposomes to estimate their affinities for cell membranes.3 The liposomes can be easily separated from the external medium by centrifugation, because they have a dense internal aqueous phase. We succeeded in finding that the amount of the polyphenols such as caffeic acid esters incorporated into the lipid bilayers governed the dose-dependency in their activities found in in vitro experiments. In this study with our liposomal systems, we examined the interaction of tea catechins with the lipid bilayer, which might govern their structure-activity relationship.

In a typical incorporation experiment, 100 mg of phosphatidyl choline from egg yolk (egg PC) from Nippon Fine Chemicals (Takasago, Hyogo, Japan), was dissolved in a small amount of chloroform. The solution was put in a round-bottomed flask and the solvent evaporated off with a rotary evaporator. The thin film of egg PC on the inner surface of the flask was dried with a vacuum pump. Then, 300 mm aqueous glucose solution (10 ml) was poured into the flask and the mixture was sonicated in an ultrasonicator. The resulting solution of multilamellar vesicles (MLV) was transferred into a 50-ml plastic centrifuge tube. The sealed tube was sonicated with a cup-horn type sonicator for 10 min at 30% of the duty cycle. The liposomal solution was diluted 10 times with phosphate-buffered saline (PBS, pH 7.4), and untrapped glucose was removed by centrifugation at 130,000 g for 5 min at 20°C. The liposomes in the sediment were suspended in PBS. The final concentration of egg PC in the liposomal solution was adjusted to 1 mg/ml after measurement of its concentration with a test kit purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Tea catechins were prepared according to our previous paper.9 A catechin solution in PBS (0.2 ml) was added to 1.8 ml of the liposome solution in PBS and the mixture was vortexed in a centrifuge tube. The final concentration of the catechin was 0 mm, 25 mm, 50 mm, or 75 mm. After incubation for 20 min at 20°C, the mixture was centrifuged at 130,000 g for 5
The amount incorporated increased with an increase in the amount added to the liposomal solution in the range tested. The amount of ECg incorporated was highest, followed by EGCG and EC, whatever the amount added. No EGCG was detected in the range tested. The partition coefficients of catechins in n-octanol/PBS decreased in the same order (Table 1). These results indicate that the affinity of each catechin for the lipid bilayer mainly reflects its hydrophobicity. The lower affinity of EC and EGCG than that of the corresponding gallic acid ester, ECg and EGCG, implies that the presence of the ester bond contributes to the higher hydrophobicity of ECg and EGCG. The affinity of ECg was higher than that of EGCG, and the affinity of EC was higher than that of EGCG. This indicates that three hydroxy groups in the B-ring of galloallocatehins such as EGCG and EGCG lowered their hydrophobicity and consequently their affinity for lipid bilayers in comparison with EC and ECg, which have two hydroxy groups in the B-ring.

Kitano et al. investigated the location of EGCG incorporated into MLV by fluorescence quenching of 2-anthroyloxy stearic acid (2-AS) and 10-anthroyloxy stearic acid (10-AS).9 The fluorescing anhydrol group of 2-AS was located in the surface region of the lipid bilayer, while that of 10-AS in the hydrophobic core of the lipid bilayer. They concluded that EGCG was located in the surface region of the lipid bilayer, because EGCG quenched more strongly the fluorescent emission of 2-AS than that of 10-AS. We attempted to confirm this effect with our SUV system, but failed, because the liposomes aggregated after addition of catechins. Then, we developed an MLV system, in which the aggregation did not occur and the fluorescence of catechins in the external medium could be eliminated by centrifugation. Consequently, we were able to compare the effects of the four catechins. In the presence of 2-AS or 10-anthroyloxy stearic acid (12-AS), 100 µg of egg PC was dissolved in a small amount of chloroform. The solution was put in a round-bottomed flask and the solvent evaporated off with a rotary evaporator. The thin film of egg PC on the inner surface of the flask was dried with a vacuum pump. Then, 300 mM aqueous glucose solution (10 ml) was poured into the flask and the mixture was vortexed for 5 min. MLV was prepared in the solution by freezing and thawing repeatedly. The liposome solution was diluted 10 times with PBS, and untrapped glucose was removed by centrifugation at 130,000 g for 5 min at 20°C. The liposomes in the sediments were suspended in PBS. The final concentration of egg PC in the liposome solution was adjusted to 1 mg/ml. After incubation with a catechin for 10 min, the MLV was purified by centrifugation and suspended with PBS. The fluorescence of 2-AS (excitation: 362 nm, emission: 446 nm) or 12-AS (excitation: 381 nm, emission: 446 nm) in the lipid bilayer was measured. The fluorescence intensity was calculated as percent of the fluorescence intensity of 2-AS or 12-AS in the absence of catechins. Figure 3 shows that the inhibiting effect of ECg on the fluorescence of 2-AS was highest, followed by those of EGCG and EC. On the other hand, EGCG had no inhibitory effect on the fluorescence of 2-AS and no catechins had any inhibito-

![Graph showing dose effects of tea catechins on the amount incorporated into the liposomes.](image)

**Fig. 2.** Dose Effects of Tea Catechins on the Amount Incorporated into the Liposomes.

A catechin in 0.2 ml of PBS was added to 1.8 ml of liposomal (SLV) solution and the mixture incubated for 20 min at 20°C. After centrifugation of the mixture, the amount of the catechin incorporated into the liposomes was measured by HPLC. ECg, ○; EGCG, ●; EGCG, △; EC, ▲; EGCG. The results are expressed as mean values of four independent experiments with the SD.

<table>
<thead>
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<th>Tea catechins</th>
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<td>ECg</td>
<td>23.9±0.3</td>
</tr>
<tr>
<td>EGCG</td>
<td>16.0±0.2</td>
</tr>
<tr>
<td>EC</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>EGCG</td>
<td>0.5±0.1</td>
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Each catechin solution in PBS (200 µM, 3 ml) was vigorously mixed with 3 ml of n-octanol. After centrifugation at 1000 r.p.m. for 10 min, the amount of the catechin in each layer was measured by HPLC. Each partition coefficient (Kb) was then calculated. The results are expressed as mean values of four independent experiments with the SD.

min. After being suspended with PBS and centrifuged again, the liposomes containing the catechin in the lipid bilayer were dissolved with 1 ml of ethanol and 1 ml of water. The amount of catechin in the solution was measured by HPLC with a UV detector using a Capcell Pak C18 UG120 column (Shiseido Co. Ltd., Tokyo, Japan), with an aqueous solution containing 18% methanol as a solvent for EC and EGCG or 25% methanol for EGCG and ECg in the presence of 0.1% trifluoroacetic acid at a detection wavelength of 285 nm. The amount of the catechin incorporated into the liposomes was then calculated. Figure 2 shows the typical dose effects of catechins on the amount incorporated. The ratio (%) of each catechin incorporated into the lipid bilayers at a certain concentration can be calculated as follows.

100 × [the amount incorporated]/[the amount added]

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**Table 1.** Partition Coefficients of Tea Catechins Evaluated with an n-Octanol/PBS System

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ry effect on the fluorescence of 12-AS. These results indicate that ECg, EGCg, and EC, located in the surface of the lipid bilayer, inhibited the fluorescence of 2-AS and that the intensity of inhibition was closely correlated with the amount of compound incorporated into the lipid bilayer.

The membrane structure may be altered by the catechins incorporated into the lipid bilayer. EGCg has been reported to induce carboxylfluorescein leakage from the liposome.9,10 This implies that EGCg at a high concentration disrupts the membrane structure. We confirmed the same effect of EGCg and ECg with our liposome system (data not shown). Furthermore, we found that EGCg and ECg at a low concentration prevented calcein (2',7'-bis(carboxyethyl)-amino-methyl)-fluorescein) leakage from SUV. Egg PC was sonicated in the presence of 300 mM glucose and 100 mM calcein to prepare SUV with a dense internal aqueous phase containing calcein.11 The after procedures of liposome purification were the same as described in the incorporation experiment. Designated amounts of catechins were added to the liposome solution, and the solution was incubated at 20°C for one hour. The external medium of the liposomes was collected by centrifugation at 130,000 g for 5 min. Fluorescence of calcein leaking from the internal aqueous phase to the external medium was measured with excitation at 488 nm and emission at 520 nm. The degree of calcein leakage was calculated as percent of the fluorescence intensity of completely released calcein from the liposomes after treatment with Triton X-100 (0.3 wt%). We observed that about 21% of calcein leaked from the liposome even without addition of catechins. Up to 1 nM, EGCg and ECg dose-dependently inhibited the leakage, but neither EC nor EGC did (Fig. 4). Judging from these results, we suppose that EGCg and ECg made the membrane structure tight and sealed the gaps through which calcein leaked. Thus, EGCg and ECg at a low concentration perturbed the membrane structure.

The order of activity of various tea catechins reported previously are as follows. Growth inhibition of human lung cancer cell line: ECG > EGCg > EGCG > EC;12 Inactivation effect on human type-A influenza virus, EGC > EGCg > EGCG;23 Inhibitory effects on the oxidative modification of low density lipoprotein, EGCg > ECG > EGC > EC or EGCG > EGCG > EGCG;12,34 Antibacterial activity against Clostridium botulinum, EGCg > ECG > EGC > EC.35 These results indicate that the activities of ECg and EGCg were always higher than those of EC and EGC. Phospholipids are the major components of cell membranes, envelopes of viruses, and surface region of low density lipoproteins. Considering these facts and our results here, we conclude that the presence of gallic acid esters in the structures of ECg and EGCg is responsible for their high affinity for the lipid bilayers and high amounts incorporated, which affect the membrane structure and partly account for the high activities in the in vitro experiments. Vinson et al. reported that lipoprotein-bound antioxidant activity of EGCg was higher than that of EC.12 EGCg could also bind to apolipoprotein. Therefore, further investigation is necessary to clarify the part in a lipoprotein to which catechins bind.

Acknowledgment

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References


