Effects of External Factors on the Interaction of Tea Catechins with Lipid Bilayers

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Green tea contains a high concentration of such catechins as (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), and (−)-epigallocatechin gallate (EGCg). Their biological activities have been evaluated by in vitro experiments using cultured cells or bacteria, but the order of activity of the various catechins differed with the study. We have been studying the interaction of tea catechins with lipid bilayers, and clarified that the number of hydroxyl groups on the B-ring, the presence of the galloyl moiety, and the stereochemical structure of each catechin govern their affinity for lipid bilayers. We investigated in this present study the effects of various external factors on the affinity of tea catechins for lipid bilayers by using liposomes as model membranes. The amount of tea catechins incorporated into the lipid bilayers increased with increasing salt concentration in an aqueous medium and decreased with increasing negative electric charge of the lipid bilayers. Furthermore, the amount of EGCg or ECg incorporated into the lipid bilayers increased with increasing EC concentration. These results reveal that the salt concentration in an aqueous medium, the electric charge of the membrane, and the presence of other catechins governed the affinity of tea catechins for the lipid bilayers.

Key words: chemical factor; lipid bilayer; liposome; tea catechin

Green tea obtained from the leaves of the Camellia sinensis plant is one of the most popular beverages in the world. The major polyphenolic compounds in green tea are catechins, including (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), and (−)-epigallocatechin gallate (EGCg) (Fig. 1). Their composition varies depending on the season of harvest and manufacturing process, green tea leaves usually containing about 1% EC, 2–3% EGC, 1–2% ECg, and 5–8% EGCg. The biological effects of tea and tea polyphenols such as the antioxidative action on low-density lipoprotein, anticarcinogenicity, and antibacterial activities have been examined by many in vitro and in vivo experiments. We have postulated that the difference in the activities of catechins in these experiments was partly based on the amount of catechins incorporated into the cell membrane.

The interaction of catechins with the cell or bacterial membrane has been reported by various methods. However, conventional methods can’t accurately measure the amount of a polyphenol incorporated into cells or bacteria, because some compounds are immediately metabolized. We have previously reported a new method for accurately measuring the amount of polyphenols incorporated into the lipid bilayers of liposomes to estimate their affinity for cell membranes. We examined the interaction of four catechins with lipid bilayers and found that the order of the amount of tea catechins incorporated into the lipid bilayer is (−)-epicatechin < (−)-epicatechin gallate < (−)-epigallocatechin < (−)-epigallocatechin gallate.
incorporated into the lipid bilayers was the same as that of the partition coefficients in an n-octanol/ phosphate-buffered saline (PBS) system. Furthermore, tea catechins with a galloyl moiety were located on the surface of the lipid bilayers, and perturbed the membrane structure. We found that the affinity of tea catechins for lipid bilayers was governed by the number of hydroxyl groups on the B-ring of catechin (e.g., EC > EGC; ECg > EGCg), the presence of a galloyl moiety of catechin (e.g., ECg > EC; EGCg > EGC), and the stereochemical structure of catechin (e.g., EGC > (−)-catechin gallate; EGCg > (−)-gallocatechin gallate). We investigate in this study the external factors affecting the amount of tea catechins incorporated into the lipid bilayers of liposomes; that is, the salt concentration of the medium, the electric charges of the lipid bilayers, and the presence of other catechins.

Materials and Methods

Materials. (−)-EC, (−)-EGC, (−)-ECg, and (−)-EGCg were kindly provided by Tokyo Food Techno Co. Ltd. (Shizuoka, Japan). n-Octanol was purchased from Kanto Chemical Co. (Tokyo, Japan), and phosphatidyl choline from egg yolk (egg PC) was obtained from Nippon Fine Chemicals (Hyogo, Japan). Phosphatidyl serine (PS), dicetyl phosphate (DCP), and stearylamine (SA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A).

Salt concentrations obtained by diluting PBS. PBS (pH 7.4) diluted 1, 2 or 4 fold with water was used as PBS, 1/2PBS, and 1/4PBS, respectively, and contained 0.9%, 0.45% and 0.225% NaCl, respectively.

Incorporation into lipid bilayers. The affinity of each compound for lipid bilayers was measured as previously reported. The thin film of egg PC in the presence or absence of 10% PS, DCP or SA on the inner surface of the flask was dried with a vacuum pump. A 300 μl aqueous glucose solution was then poured into the flask, and the mixture was sonicated in an ultrasonicator. The resulting solution of multilamellar vesicles was then sonicated in a cup-horn type of sonicator to change the multilamellar vesicles to small unilamellar vesicles. The liposomal solution was diluted 10 fold with PBS, 1/2PBS or 1/4PBS. The final concentration of egg PC in the liposomal solution was adjusted to 1 mg/ml. Each catechin (100 nmol) solution in PBS was added to the liposomal solution. When investigating the effect of other catechins, we used the catechin whose amount incorporated into the lipid bilayers was to be measured at a final concentration of 100 nmol, and changed the concentrations of the other catechins to 0, 25, 50, 100, 150 or 200 nmol. The amount of catechin in the solution was measured by HPLC with a UV detector, using a Capcell Pak C18 UG120 column (Shiseido Co., Tokyo, Japan). The mobile phase was 18% methanol for EC and EGC, and 25% methanol for ECg and EGCg, with water in the presence of 0.1% trifluoroacetic acid, the detection wavelength being 285 nm. The proportion (%) of each catechin incorporated into the lipid bilayers was calculated as follows:

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\text{[amount incorporated]/[amount added]} \times 100.
\]

Partition coefficients. Each catechin solution in PBS (2 ml) was vigorously mixed with 2 ml of n-octanol. After centrifugation at 200 g for 10 min, the amount of the catechin in each layer was measured by HPLC. The partition coefficient of each catechin was then calculated.

Fluorescence intensity. Fluorescence spectra were recorded with a Hitachi F-3010 fluorescence spectrophotometer. The concentration of the catechin whose fluorescence intensity was to be measured was 200 nmol, and that of the other catechins was changed to 0, 100, 200 and 400 nmol. The excitation and emission wavelengths were 231 nm, and 321 nm, respectively, for ECg, and 275 nm and 373 nm, respectively, for EGCg. All the sample solutions were purged with N2 gas before the fluorescence measurement.

Results and Discussion

Effect of the salt concentration in the aqueous medium of the liposome solutions

The amount of catechins incubated with the liposomes and centrifuged once was almost same as that centrifuged twice (data not shown). Therefore, the interaction of the catechins with the membrane surface was not weak binding, and catechins could be incorporated into the lipid bilayers. The amounts of EC, ECg and EGCg incorporated into the lipid bilayers increased as the salt concentration was increased (Table 1). EGC was not incorporated into the lipid bilayers in the range tested. The partition coefficient of each catechin evaluated with an n-octanol/PBS system also increased as the salt concentration increased (Table 1). These results suggest that the partition coefficient and the affinity of the catechins for lipid bilayers strongly depended on the salting-out effect of the aqueous medium. Thus, the in vitro activities of a catechin can be expected to depend on the salt concentration of the medium.

Effect of the electric charge of the membrane

The antibacterial activity of catechins is higher against Gram-positive bacteria than that against Gram-negative bacteria. The outer membrane of Gram-positive bacteria consists of a thick peptidogly-
The lipid bilayers. We used 10 lipopolysaccharides. To verify their explanation di-attributable to the presence of negatively charged resistance of Gram-negative bacteria to EGCg was amounts incorporated into the lipid bilayers containing (Table 2). On the other hand, their tinctly lower than those incorporated into the control liposomes (Table 2). On the other hand, their discharge, and those containing SA have a positive liposomes containing PS or DCP have a negative not have a net electric charge. On the other hand, the control liposomes prepared from PC alone do Prepare PC membranes with various electric charges. The control liposomes prepared from PC alone were used as the control. The liposomes containing PS or DCP had a negative charge, and the liposomes containing SA had a positive electric charge. Each result is shown as the mean value of four independent experiments. Table 2. Amount of Tea Catechins Incorporated into the Lipid Bilayers and Partition Coefficients of Tea Catechins in Various Concentrations of PBS

<table>
<thead>
<tr>
<th>Tea catechin</th>
<th>Incorporation into lipid bilayers (%)</th>
<th>Partition coefficient (log P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/4 PBS</td>
<td>1/2 PBS</td>
</tr>
<tr>
<td>EGC</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>EC</td>
<td>9.2 ± 0.4</td>
<td>16.3 ± 0.6</td>
</tr>
<tr>
<td>EGCg</td>
<td>38.0 ± 0.0</td>
<td>44.5 ± 0.8</td>
</tr>
<tr>
<td>ECg</td>
<td>53.2 ± 0.4</td>
<td>56.6 ± 0.0</td>
</tr>
</tbody>
</table>

* A catechin (100 nmol) in 0.2 ml of PBS at various concentrations was added to 1.8 ml of the liposomal solution. The mixture was incubated at 20°C for 20 min and then centrifuged. The proportion (%) was calculated by dividing the amount incorporated by the amount added. Each result is shown as the mean value ± SD of four independent experiments.

* The partition coefficient was calculated by dividing the amount of each catechin in n-octanol (2 ml) by that in each buffer (2 ml) after vigorously mixing and centrifuging a mixture containing each catechin at a concentration of 50 μM. Each result is expressed as the common logarithm, and is shown as the mean value ± SD of four independent experiments.

can layer. On the other hand, Gram-negative bacteria have an external membrane consisting of a phospholipid and lipopolysaccharide outside a thin peptidoglycan layer. Ikigai et al. have reported that EGCg caused leakage of 5,6-carboxyfluorescein, a fluorescence substance, entrapped in the intraliposomal space and that the amount of 5,6-carboxyfluorescein that leaked from the liposomes with a negative charge was lower than that leaking from the control liposomes.19 They explained that the resistance of Gram-negative bacteria to EGCg was attributable to the presence of negatively charged lipopolysaccharides. To verify their explanation directly, we investigated the effect of the electric charge of the membrane on the affinity of tea catechins for the lipid bilayers. We used 10% PS, DCP or SA to prepare PC membranes with various electric charges. The control liposomes prepared from PC alone do not have a net electric charge. On the other hand, the liposomes containing PS or DCP have a negative charge, and those containing SA have a positive charge. The amounts of ECg and EGCg incorporated into the lipid bilayers containing PS or DCP were distinctly lower than those incorporated into the control liposomes (Table 2). On the other hand, their amounts incorporated into the lipid bilayers contain-
Fig. 2. Effects of Tea Catechins on the Amount Incorporated into Lipid Bilayers in the Presence of Other Catechins.
Each catechin (100 nmol) and an other catechin solution in PBS were added to the liposome solution. The mixture was incubated at 20°C for 20 min and centrifuged. The proportion (%) was calculated by dividing the amount incorporated by the amount added. Each result is shown as the mean value ± SD of four independent experiments. (A) EGC, (B) EC, (C) EGCg, and (D) ECg; ● EC, ○ EGC, ▲ ECg, and △ EGCg.

Fig. 3. Partition Coefficients of Tea Catechins in the Presence of Other Catechins.
The partition coefficient was calculated by dividing the amount of each catechin in n-octanol (2 ml) by that in PBS (2 ml) after vigorously mixing and centrifuging a mixture containing each catechin at a concentration of 50 μM. Each result is shown as the mean value ± SD of four independent experiments. (A) EGC, (B) EC, (C) EGCg, and (D) ECg; ● EC, ○ EGC, ▲ ECg, and △ EGCg.
Fig. 4. Fluorescence Intensity of Tea Catechins in the Presence of Other Catechins.

Each catechin (200 nmol) and an other catechin (0, 100, 200, 400 nmol) were mixed, and the fluorescence intensity was measured. Each result is shown as the mean value ± SD of four independent experiments. (A-1) EGCg solution in methanol, (A-2) EGCg solution in water, (B-1) ECg solution in methanol, and (B-2) ECg solution in water; • EC, ○ EGC, ▲ ECg, and △ EGCg.

by EGCg and that the [3H] EGCg incorporation into PC-9 cells was significantly enhanced by EC.23,24) Our present results indicate that the enhancement of EGCg incorporation into the cells by EC should involve a simple physico-chemical process.

EGCg tends to aggregate in an aqueous solution.13) We presume that EC destroys the aggregate to more uniformly disperse EGCg molecules in the medium. Generally, the fluorescence of aggregated compounds is self-quenched and its intensity is lower than that of uniformly dispersed compounds. We measured the fluorescence intensity of each catechin in the presence of other catechins. The fluorescence spectrum of ECg or EGCg was obtained after subtracting the fluorescence spectrum of the other catechins from the total fluorescence spectrum. The fluorescence intensity of each compound in methanol was much higher than that in the aqueous solution (e.g., Figs. 4(A-1) vs. 4(A-2), and Figs. 4(B-1) vs. 4(B-2)). This indicates that the catechins tended to be more dispersed in methanol than in the aqueous solution. EC and EGC emitted fluorescence under the same conditions, and their fluorescence intensities were not changed in the presence of other catechins in the aqueous medium (data not shown). EGCg and ECg showed enhanced fluorescence intensity in the presence of EC (Figs. 4(A) and 4(B)), and their incorporation into the lipid bilayers was also enhanced (Figs. 2(C) and 2(D)). We presume that the aggregate of EGCg or ECg might have been destroyed by EC, resulting in a complex structure uniformly dispersed in the aqueous medium that provided easier access of EGCg or ECg in the complex structure to the lipid bilayers. Further studies are needed to verify these assumptions.

We have previously reported that the affinity of tea catechins for lipid bilayers was governed by the number of hydroxyl groups on the B-ring of the catechin, the presence of the galloyl moiety of the catechin, and the stereochemical structure of the catechin.19) We found in this study that the salt concentration in the aqueous medium, the electrical charge of the membrane, and the presence of other catechins also governed the affinity of tea catechins for lipid bilayers, which, in turn, could govern the results of in vitro experiments.

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References


