Direct Evidence of Interaction of a Green Tea Polyphenol, Epigallocatechin Gallate, with Lipid Bilayers by Solid-state Nuclear Magnetic Resonance

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The interaction of a tea catechin, epigallocatechin gallate (EGCg), with the model membrane of dimyrystoylphosphatidylcholine (DMPC) was studied by solid-state 31P and 2H NMR. The 31P chemical shift anisotropy of the DMPC phosphate group decreased on addition of EGCg. The 2H NMR spectrum of [4-2H]EGCg, which is deuterated at the 4-position, in the DMPC liposomes gave deuterium nuclei with much smaller quadrupole splittings than those in the solid phase. These 31P and 2H NMR observations provide direct experimental evidence that the EGCg molecule interacts with the lipid bilayers.

Key words: tea catechins; epigallocatechin gallate; liposome; lipid bilayer; solid-state NMR

Catechins are a group of polyphenols that occur naturally in certain species of plants, including tea (Camellia sinensis), and are major ingredients in green tea infusions. Recently, they have been found to have various biological effects such as antimitogenicity,1,2) antitumorigenicity,3) antimitogenicity,4,5) and antioxidant,6–8) antihypercholesterolemia,9) and antibacterial activities.10–13)

The biological activities of catechins have been evaluated by in vitro experiments using cultured cells or bacteria, but the order of activity was variable. The differences in biological activities may be attributed partly to the amounts of compounds incorporated into the lipid bilayers. Measurements of the amount of a polyphenol incorporated into cells are not always accurate, because some compounds are metabolized immediately. Hence, an accurate method to measure the amount of compound incorporated into model membranes such as liposomes is needed. We developed a method of estimating the affinity of polyphenols for cell membranes.14) We examined the interaction of four catechins, i.e., epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate (EGCg: 1), with lipid bilayers,15–17) and found that the order of the amount of tea catechins incorporated into liposomes was the same as that of the partition coefficients in a 1-octanol/phosphate-buffered saline system. We found that the affinity of tea catechins for lipid bilayers is governed by the number of hydroxyl groups on the B-ring, the presence of the gallol moiety, and the stereochromophic structure of each catechin.15,16) Furthermore, the salt concentration in an aqueous medium, the electric charges of the membrane, and the presence of other catechins were also important factors in the affinity of tea catechins for lipid bilayers.17)

These experimental findings suggested that interaction of catechins with the phospholipid domain of membranes occurs. But interaction of catechins with membranes has not been observed directly, and the site and the orientation of catechins in membranes of phospholipids have remained undiscovered.

Solid-state NMR is one of the most promising techniques for analyzing the interaction of drugs or xenobiotics with membranes due to its ability to elucidate the extent or the manner of binding to lipids, the phase behavior, the dynamic feature of bound drugs, and so on. We developed a method of estimating the affinity of polyphenols for cell membranes.14) We examined the interaction of four catechins, i.e., epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate (EGCg: 1), with lipid bilayers,15–17) and found that the order of the amount of tea catechins incorporated into liposomes was the same as that of the partition coefficients in a 1-octanol/phosphate-buffered saline system. We found that the affinity of tea catechins for lipid bilayers is governed by the number of hydroxyl groups on the B-ring, the presence of the gallol moiety, and the stereochromophic structure of each catechin.15,16) Furthermore, the salt concentration in an aqueous medium, the electric charges of the membrane, and the presence of other catechins were also important factors in the affinity of tea catechins for lipid bilayers.17)

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and perturbation of the organization of membrane lipids.\textsuperscript{18}) In this study, we investigated the interaction of EGCg with dimyristoylphosphatidylcholine (DMPC) bilayers by solid-state \textsuperscript{31}P and \textsuperscript{2}H NMR spectroscopy. \textsuperscript{31}P NMR of the phosphocholine head-group of DMPC provided information about the behavior of the surrounding bilayers when EGCg interacts. Further, the orientational and motional properties of \textsuperscript{[4-\textsuperscript{2}H]}EGCg, deuterium-labeled at the 4-position of EGCg, interacting with DMPC liposomes, were analyzed by solid-state \textsuperscript{2}H NMR.

Materials and Methods

Materials. \textsuperscript{[4-\textsuperscript{2}H]}EGCg deuterated at the 4-position in the C ring was synthesized according to the scheme reported previously.\textsuperscript{19}) The 4-position of EGCg was selected as the labeling position of deuterium to avoid hydrogen exchange. DMPC was purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). Deuterium-depleted water was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin, U.S.A.).

Sample preparation. For \textsuperscript{31}P NMR measurements, 5 mg of EGCg (1) and 148 mg of DMPC were dissolved in chloroform:methanol (1:1, v/v) with an EGCg-to-DMPC molar ratio of 1:20, and the solvent was subsequently evaporated in vacuo, followed by hydration with 1 ml of Tris buffer (20 mm Tris, 100 mm NaCl, and pH 7.5). For \textsuperscript{2}H NMR measurements, \textsuperscript{[4-\textsuperscript{2}H]}EGCg (2) and deuterium-depleted water were used instead of EGCg and Tris buffer respectively. A freeze-thaw cycle was repeated several times until homogeneous multibilayer liposomes were obtained. The diameter of the vesicles obtained by this procedure was 200–5000 nm.\textsuperscript{20}) These samples were placed in glass sample tubes and sealed with glue to prevent dehydration.

Measurements of \textsuperscript{31}P NMR spectra. \textsuperscript{31}P NMR spectra were recorded on a Chemagnetics CMX-400 NMR spectrometer operating at a resonance frequency of 161.99 MHz under static or magic angle spinning (MAS) conditions. A single-pulse excitation method with dipolar decoupling was used to obtain \textsuperscript{31}P NMR spectra using a recycle delay of 2 s and a proton decoupling field of 50 kHz. The number of scans was typically 1,200. Spinning frequencies were adjusted to 4,000 ± 4 Hz for the MAS experiments. An exponential multiplication with 20 Hz Lorentzian line broadening was applied to all \textsuperscript{31}P free induction decays prior to Fourier transformation. Chemical shifts of 85% H\textsubscript{3}PO\textsubscript{4} served as the reference for \textsuperscript{31}P NMR spectra.

Measurements of \textsuperscript{2}H NMR spectra. \textsuperscript{2}H NMR spectra were recorded on a Chemagnetics CMX-400 NMR spectrometer operating at a resonance frequency of 61.4 MHz using the quadrupole echo pulse sequence (90°–τ–90°–τ–echo) at 30°C.\textsuperscript{21}) Typical acquisition parameters were a 90° pulse length of 2.5 μs, a pulse interval of 60 μs, and a recycle delay of 500 ms. The number of scans was typically 180,000. The time domain data were left-shifted to the echo maximum, and Lorentzian line broadening of 2 kHz was applied before Fourier transformation.

Results and Discussion

Figure 2a shows that the \textsuperscript{31}P NMR spectra of EGCg interacted with DMPC bilayers at temperatures between 40°C and 10°C, together with the spectra of DMPC bilayers without EGCg in the right panel (Fig. 2b). An axially symmetrical powder pattern, which is character-
interaction of the liquid crystalline phase, was observed irrespective of the phase transition temperature at 24 °C (T_{m}) of the pure DMPC bilayers. It is notable that the ^{31}P chemical shift anisotropy of DMPC in the presence of EGCg decreased as compared with that without EGCg (Table 1) at the same temperature. Further, the δ_{iso} chemical shift values obtained in the MAS experiments also indicated differences between the ^{31}P NMR spectra with and without EGCg (Table 2). These findings suggest that EGCg interacts with the lipid bilayers and affects the head-group motion of the phospholipids. Previously, we stated that the EGCg is assumed to be located in the surface of the lipid bilayers by fluorescence quenching of 2-(9-anthroyloxy) stearic acid (2-AS) and 12-AS.16) The present ^{31}P NMR study also indicates that EGCg is located in the head-group region of the phospholipids in the lipid bilayers.

Figure 3 shows the ^{2}H NMR spectrum of [4-^{2}H]EGCg in the solid phase. On the other hand, Fig. 4 shows the ^{2}H NMR spectrum of [4-^{2}H]EGCg in the DMPC bilayer. The observed ^{2}H spectrum in the DMPC bilayers consists of two components (Fig. 4). The central line arises from the residual HDO signal or free EGCg molecule in aqueous solution. The other component shows the quadrupole splitting, indicating that EGCg interacts with the DMPC bilayers. The observed ^{2}H quadrupole splitting (Dq) of [4-^{2}H]EGCg interacting with the DMPC bilayer, 30 kHz (Fig. 4), is much smaller than that in the solid state 125 kHz (Fig. 3). Obviously, such a reduction in quadrupole splitting is associated with the presence of accompanying large amplitude motions of EGCg within the lipid bilayer.

In general, the ^{2}H quadrupole splitting Dq in a certain direction of the C–^{2}H band is related to the angle of the principal axis of the electric field gradient tensor (C–^{2}H vector) with respect to the applied magnetic field (θ):

\[ Dq = \frac{3}{4} \left( \frac{e^{2}qQ}{h} \right) (3\cos^{2}\theta - 1) \]  

where \( e^{2}qQ/h \) is the quadrupole coupling constant, 167 kHz for the sp\(^{3}\) hybridized C–^{2}H pair.22) For rigid polycrystalline solids, all values of \( \theta \) are possible. We obtained a powder pattern ^{2}H NMR spectrum with a splitting of 125 kHz for the perpendicular component. The ^{2}H NMR spectrum of [4-^{2}H]EGCg in the solid state gave rise to the characteristic deuterium quadrupole splitting of about 125 kHz. On the other hand, the reduced quadrupole splittings in the ^{2}H NMR spectrum of [4-^{2}H]EGCg in the DMPC bilayer arises from motional averaged splittings due to the presence of anisotropic molecular motion as expressed by \( (3\cos^{2}\theta - 1) \) in contrast to the case of the static state in equation (1). The presence of single ^{2}H quadrupolar splitting is consistent with the previous observation for 12-O-[20-^{2}H]tetradecanoylphorbol-13-acetate in the DMPC bilayer.23) This means that there exists at least one kind of EGCg molecule with rotational motion in the lipid bilayers. Alternatively, such splitting could be explained by the asymmetric electrical field gradient, which was created by partial molecular motion. 24) In any case, the results of ^{2}H NMR are consistent with the view that EGCg interacts with the lipid bilayers.

Interaction of tea catechins with lipid bilayers has been described in several papers. Ikigai et al. found that EGCg caused leakage of 5,6-carboxylfluorescein from phosphatidylcholine liposomes.25) They ascribed the antibacterial action of green tea to damage to bacterial

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**Table 1.** Chemical Shift Anisotropy for Phosphorus at the Head-group of DMPC

<table>
<thead>
<tr>
<th></th>
<th>EGCg/DMPC (ppm)</th>
<th>DMPC (ppm)</th>
</tr>
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<tbody>
<tr>
<td>40 °C</td>
<td>43.8(^{a})</td>
<td>45.2(^{a})</td>
</tr>
<tr>
<td>30 °C</td>
<td>44.9(^{a})</td>
<td>46.7(^{a})</td>
</tr>
<tr>
<td>20 °C</td>
<td>44.6(^{a})</td>
<td>46.6(^{a})</td>
</tr>
<tr>
<td>10 °C</td>
<td>49.8(^{a})</td>
<td>52.3(^{a})</td>
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\(^{a}\)Chemical shift difference with an uncertainty of ±0.3 ppm.

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**Table 2.** ^{31}P Isotropic Chemical Shift Values of δ_{iso} Obtained by MAS Experiments\(^{a}\)

<table>
<thead>
<tr>
<th></th>
<th>+EGCg (ppm)</th>
<th>−EGCg (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 °C</td>
<td>−0.81</td>
<td>−0.76</td>
</tr>
<tr>
<td>30 °C</td>
<td>−0.84</td>
<td>−0.80</td>
</tr>
<tr>
<td>20 °C</td>
<td>−0.89</td>
<td>−0.85</td>
</tr>
<tr>
<td>10 °C</td>
<td>−1.02</td>
<td>−0.94</td>
</tr>
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\(^{a}\)Chemical shift difference with an uncertainty of ±0.06 ppm.
membranes by catechins. The antioxidant activity of EGCg and its hydrophobic derivatives was investigated with egg PC liposomes by Tanaka et al. \(^{26}\) They studied the inhibitory activity of EGCg against lipid peroxidation caused by both water-soluble and lipid soluble radical initiators, and concluded that EGCg was present both in the water phase and in the surface of the lipid bilayers. More recently Caturia et al. also reported a relationship between the antioxidant and the antibacterial properties of galloylated catechins and the structure of phospholipid model membranes.\(^{27}\)

As shown by their studies, the biological activities of tea catechins are related to the interaction of the lipid membranes. But in spite of speculation that tea catechins interact with the lipid bilayers, there has been no direct experimental evidence. The present study provided direct experimental evidence of interaction of EGCg with the lipid bilayers by solid-state \(^{31}\)P and \(^{2}H\) NMR. \(^{31}\)P NMR gave information about the location of EGCg in lipid bilayers and the perturbation of the membrane structure. Further, the small quadrupole splitting (about 30 kHz) of the \(^{2}H\) NMR spectrum of \(^{[4-\text{H}]\text{EGCg}}\) in DMPC liposomes indicated that EGCg was present in the lipid bilayers. The present study provides some insight into this mechanism for understanding the interaction between tea catechins and membranes.

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**References**


