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Bactericidal catechins damage the lipid bilayer

Hajime Ikigai [a,c], Taiji Nakae [a], Yukihiko Hara [b] and Tadakatsu Shimamura [c]

[a] *Department of Molecular Life Science, Tokai University School of Medicine, Isehara (Japan),* [b] *Food Research Laboratories, Mitsui Norin Co., Fujieda (Japan) and* [c] *Department of Microbiology and Immunology, Showa University School of Medicine, Tokyo (Japan)*

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The mode of antibacterial action of, the green tea (*Camellia sinensis*) extracts, (−)-epigallocatechin gallate (EGCg) and (−)-epicatechin (EC) was investigated. Strong bactericidal EGCg caused leakage of 5,6-carboxyfluorescein from phosphatidylcholine liposomes (PC), but EC with very weak bactericidal activity caused little damage to the membrane. Phosphatidylserine and dicetyl phosphate partially protected the membrane from EGCg-mediated damage when reconstituted into the liposome membrane with PC. EGCg, but not EC, caused strong aggregation and NPN-fluorescence quenching of PC-liposomes and these actions were markedly lowered in the presence of negatively charged lipids. These results show that bactericidal catechins primarily act on and damage bacterial membranes. The observation that Gram-negative bacteria are more resistant to bactericidal catechins than Gram-positive bacteria can be explained to some extent by the presence of negatively charged lipopolysaccharide.

Introduction

Tea powder or tea extracts showed growth inhibition of both Gram-positive and Gram-negative bacteria [1–3], when added into the bacterial culture medium. Mammalian viruses treated in vitro with tea extracts showed reduced infectivity to cultured cells or embryonated egg [4–6]. Polyphenols extracted from tea and coffee, collectively termed catechins, showed similar antimicrobial effects [7–9]. Catechins acted on microbes, but also on enzymes [10,11], and hemolytic toxin [12], indicating that the compounds deactivate proteins. Catechins also exert their effect on mammalian cells, inducing lymphocyte proliferation, immunoglobulin synthesis [13], and mitogenicity of B-lymphocytes [14], and stimulating the interleukin production of human leukocytes [15] at a low concentration. These biological activities correlate with the presence of galloyl and gallic moieties of the catechin structure (Fig. 1). (−)-Epigallocatechin gallate (EGCg) has the strongest biological activity and (−)-epicatechin (EC) has the least [7].

The bactericidal activity of catechins appears higher against Gram-positive bacteria compared with that against gram-negatives. This was attributed to the functional barrier present on the outer membrane. Gram-negative bacteria have the outer membrane exterior to the cytoplasmic membrane, thus forming a tight diffusion barrier against hydrophobic compounds and hydrophilic compounds of large size, i.e., $M_r > 600$, in the outer membrane of *Escherichia coli* [16]. The mode of catechin action was explained by suggesting that catechins exert an effect on the membrane, changing the latter's fluidity [17], morphology [18], and decreasing the flux of thiourea and cycloleucine [17]. The immuno-enhancing activity of catechin is thought to be due to the catechin action on the plasma membrane of the target cell [13].

To investigate the mechanism of the bactericidal properties of catechins, we studied the action of catechins on liposomal and bacterial membranes. This paper reports our finding that the bactericidal catechin disrupts the membrane.

Materials and Methods

Chemicals

5,6-Carboxyfluorescein (CF) was obtained from Eastman Kodak (Rochester) and used without further

Correspondence to: T. Nakae, Department of Molecular Life Science, Tokai University School of Medicine, Isehara 259-11, Japan.
Abbreviations: EGCg, (−)-epigallocatechin gallate; NPN, *N*-phenyl-1-naphthylamine; EC, (−)-epicatechin; PC, phosphatidylcholine; PS, phosphatidylserine; CF, 5,6-carboxyfluorescein; DCP, dicetyl phosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SA, stearylamine.

purification. Egg-yolk phosphatidylcholine (PC) and bovine brain phosphatidylserine (PS) were purchased from Sigma (St. Louis). Dicetyl phosphate (DCP) and stearylamine (SA) were obtained from Wako (Osaka).

Preparation of catechins

EGCg and EC were purified as described previously [15]. Briefly, green tea (*Camellia sinensis*) (100 g) was suspensed in 1000 ml of hot water. A crude catechin mixture was extracted from the water-soluble fraction of green tea with chloroform and ethyl acetate. After concentrating and lyophilizing the catechin mixture in the ethyl acetate soluble fraction, EGCg and EC were purified by the high performance liquid chromatography using a Waters Prep PAC-500/C_{18} column (5 cm × 30 cm) equilibrated with tetrahydrofuran/acetone/H_2O, as described previously [3].

Bacterial strains and culture conditions

Bacterial strains used were *Escherichia coli* K-12 strain G6 and *Staphylococcus aureus* ATCC25932. Bacterial cells were grown overnight in 10 ml of L-broth containing 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl per liter (pH 7.2) with shaking at 100 rpm at 37°C. The culture was diluted with 90 ml of prewarmed medium and the mixture was shaken at 240 rpm for 3.5 h at 37°C. Cells were harvested, washed once with 150 mM NaCl/5 mM Hepes buffer (pH 7.2) and resuspended in the same buffer at the absorbance of 0.5 at 600 nm.

Antimicrobial susceptibility test

The minimal growth inhibitory concentration (MIC) of catechins was determined by the 2-fold agar dilution method using L-broth supplemented with 1.5% agar [19].

Preparation of liposomes

PC in chloroform (7.5 μmol) was dried at the bottom of a glass tube and resuspended in 1 ml of a solution containing 150 mM NaCl and 10 mM Hepes (pH 7.2). The suspension was subjected to sonic oscillation in the presence or absence of CF for 10 min at 20 watt with a Branson Sonifier-200 equipped with a microtip [20].

TABLE I

Minimal growth inhibitory concentration of EGCg and EC

MIC was determined by the two-fold agar dilution method.

Organism	MIC (μg/ml)	
	EGCg	EC
S. aureus ATCC25932	73	183
E. coli K-12	573	>1145

CF-release measurement

This was performed as described previously [20]. The CF-encapsulated PC-liposomes were prepared by passing the liposomes prepared in the presence of CF through a Sephacryl CL-4B equilibrated with 150 mM NaCl/10 mM Hepes (pH 7.2).

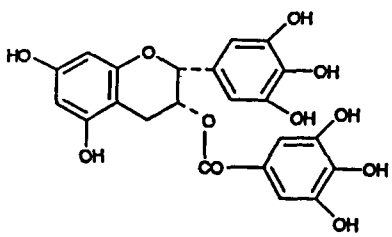
NPN-fluorescence measurements

NPN fluorescence was measured as described previously [21]. NPN was dissolved to 1 mM in acetone and 1 μl was added to 1.0 ml of the liposome suspension. This amount of acetone had no detectable effect on fluorescence measurement. Fluorescence intensity was recorded at the excitation and emission wavelengths of 370 nm and 450 nm, respectively, at a slit width of 5 nm using a Hitachi fluorescence spectrophotometer 650-10S.

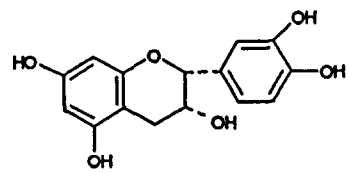
Results

Antimicrobial activity of catechins

Bacterial susceptibility to catechins was tested by determining the minimal growth inhibitory concentration (MIC) by the agar dilution method. The result that *S. aureus* was more susceptible to EGCg than *E. coli*. Thought the MIC of EC against *S. aureus* was 183 μg/ml, that against *E. coli* appeared to be undetectably high at more than 1.1 mg/ml (Table I). This



EGCg



EC

Fig. 1. Structure of EGCg and EC.

marked difference in catechin susceptibility between the Gram-positive and Gram-negative bacteria is probably attributable to the barrier function of the outer membrane.

Catechin-mediated damage of liposomal membranes

We tested the membrane damaging activity of catechins by determining the release of CF entrapped in the intraliposomal space (Fig. 2). EGCg at 1.25 mM (606 μg/ml) caused rapid leakage of CF and the extent of CF-release reached 50% within 5 min and slowly increased thereafter. As seen in Fig. 2, EGCg caused CF-release in a concentration-dependent manner. EC at 1.25 mM (363 μg/ml) caused CF-release equivalent to or less than CF-release by 0.16 mM EGCg at 5 min (Fig. 2). The extent of CF-release was nearly parallel with bactericidal activity (see Table I). These results indicate that bactericidal EGCg damages the liposome membrane and causes leakage of intraliposomal CF. Thus, it is possible that EGCg first damages the bacterial membranes resulting in leakage of intracellular materials. This membrane damage may enhance the penetration of catechin itself into the interior of the cell. Gram-negative bacteria are generally more resistant to catechins than Gram-positives, which is probably due to the tight penetration barrier of the outer membrane. Yet is is possible that the outer membrane constituents also play a role to some extent in catechins resistance.

To find out effect of the lipid composition and charge on catechin susceptibility, PC-liposomes containing, SA, DCP or PS were prepared and CF-release was monitored in the presence of catechin. The results showed that PS and DCP significantly reduced the catechin-mediated CF release, but SA had no measurable effect (Fig. 3). These results indicated that the

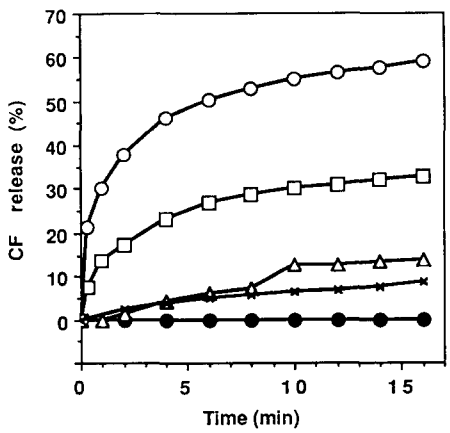


Fig. 2. Catechin-mediated CF release from the CF-encapsulated PC liposomes. Liposome suspension containing 1 nmol PC per 1.0 ml of 10 mM Hepes/150 mM NaCl (pH 7.2) was placed in a quartz cuvette. Symbols: •, without catechin; △, 0.16 mM EGCg; □, 0.625 mM EGCg; ○, 1.25 mM EGCg; ×, 1.25 mM EC.

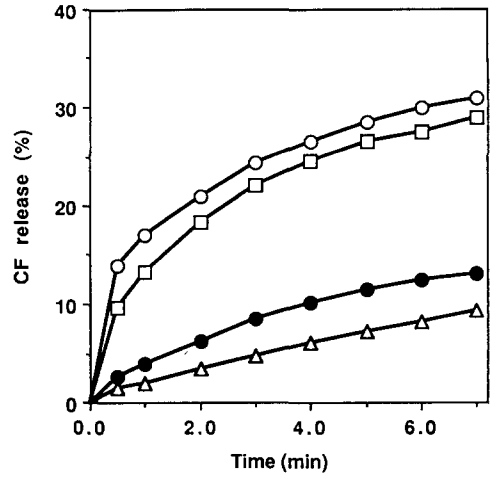


Fig. 3. Effect of lipid composition on the EGCg-mediated CF release from the CF-encapsulated liposomes. Liposome membranes were prepared from ○, PC alone, △, 90 mol% PC plus 10 mol% DCP; □, 90 mol% PC plus 10 mol% SA; •, 92.5 mol% PC plus 7.5 mol% PS. Liposome containing 1 nmol lipid per 1.0 ml of 10 mM Hepes/150 mM NaCl (pH 7.2) was placed in a quartz cuvette. EGCg concentration was 0.6 mM throughout.

surface charge of the target membrane is important in catechin susceptibility. Furthermore, they imply that the low catechin susceptibility of Gram-negative bacteria may be due partially to the presence of a strong negative charge of lipopolysaccharide at the exterior of the outer membrane. It is unexplained, however, how uncharged catechins interact with negatively charged groups of membrane lipids. The effect of PS and DCP is not simply due to conferred hydrophilicity on the membrane, since the positively charged lipid, SA, had a marginal effect on catechin-mediated membrane damage (Fig. 3).

Catechin-caused aggregation of liposomes

The bactericidal catechin showed strong membrane damaging activity as shown by CF-release. However, the mechanism of membrane damage remains obscure. To test the interaction of catechins with the liposome membrane, we have determined the fluorescence emission of a lipophilic fluorescence probe, in the preloaded membranes. When NPN was added into the liposome suspension, fluorescence emission increased abruptly to a near maximum level, indicating that NPN had entered the hydrophobic environment (Fig. 4). Upon addition of 1.25 mM EGCg into the mixture, the fluorescence intensity dropped immediately to about 40% of the maximum level. Low concentrations of EGCg had a lesser quenching effect. Since EGCg does not quench NPN-fluorescence appreciably in solution, this fluorescence quenching was attributed to the aggregation of liposomes by added EGCg (see below). Experiments performed with *E. coli* and *S. aureus* showed essentially similar results to that shown in Fig. 4 (data

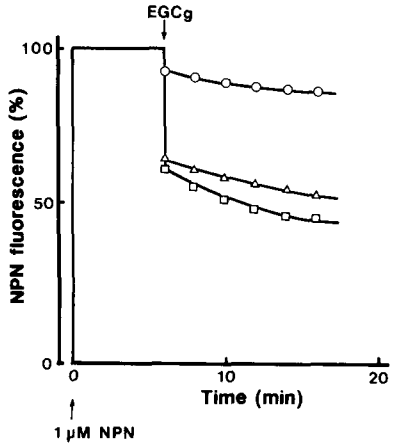


Fig. 4. Quenching of NPN fluorescence by catechins. Liposome were prepared from PC as described in Materials and Methods and the liposome suspension containing $2.5 \cdot 10^{-7}$ mol lipid in 1 ml of 10 mM Hepes/150 mM NaCl (pH 7.2) was placed in a quartz cuvette. NPN (final 10 μM) and EGCg were added at the time indicated. Symbols: ○, 0.01 mM EGCg; △, 0.16 mM EGCg; □, 1.25 mM EGCg.

not shown). The extent of fluorescence quenching by EGCg appeared more significant when the fluorescence dye was mixed with *S. aureus* than with *E. coli* (data not shown).

This observation led us to examine the action of EGCg on liposome membrane containing PC and charged lipids. The liposomes containing PC were mixed with 0.015 mM through 0.6 mM catechin, and the turbidity at 450 nm was recorded. As seen in Fig. 5A, the turbidity increased rapidly, reaching a plateau within 2 min. The absorption increment was nearly proportional to the catechin concentrations. EC showed little absorption increment even at 0.6 mM (data not shown). To see the effect of surface charge on catechin associated aggregation, liposomes containing negatively charged (DCP and PS) or positively charged lipid (SA) were prepared and tested for aggregation by EGCg. The results, shown in Fig. 5B, clearly indicated that EGCg-mediated aggregation was abolished in the liposome containing 10 mol% of PS. Similarly, EGCg-mediated turbidity increment of the 10 mol% DCP-containing liposome appeared to be lower than that of PC liposome only. Positively charged lipid, SA, at 10 mol% did not show any detectable effect on liposome aggregation. These results suggest that one of the reasons for Gram-negative bacteria being more resistant to EGCg than Gram-positive bacteria is the presence of a strong negative charge conferred by lipopolysaccharide on the surface of the Gram-negative bacteria. Tests of the intact bacteria for EGCg-mediated aggregation showed that *S. aureus* cells aggregated to a significant extent in the presence of 1.25 mM EGCg, but the aggregation was undetectably low in *E. coli* (Fig. 6).

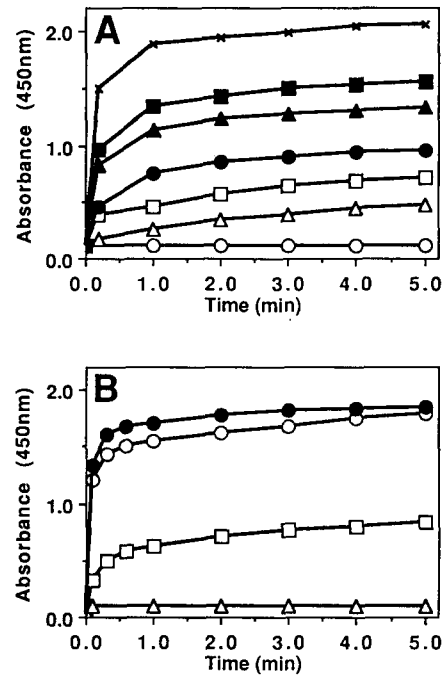


Fig. 5. Effect of lipid composition on the EGCg-mediated liposome aggregation. (A) Liposomes made of PC only. EGCg concentrations were as follows: ○, without catechin; △, 0.015 mM; □, 0.03 mM; ●, 0.075 mM; ▲, 0.15 mM; ■, 0.3 mM; ×, 0.6 mM. (B) Liposomes were constructed from PC plus PS, DCP or SA. Symbols, ○, PC alone, △, 90 mol% PC plus 10 mol% PS; □, 90 mol% PC plus 10 mol% DCP; ●, 90 mol% PC plus 10 mol% SA. Total liposome concentration was 1 nmol lipid per ml throughout. EGCg concentration was 0.6 mM throughout.

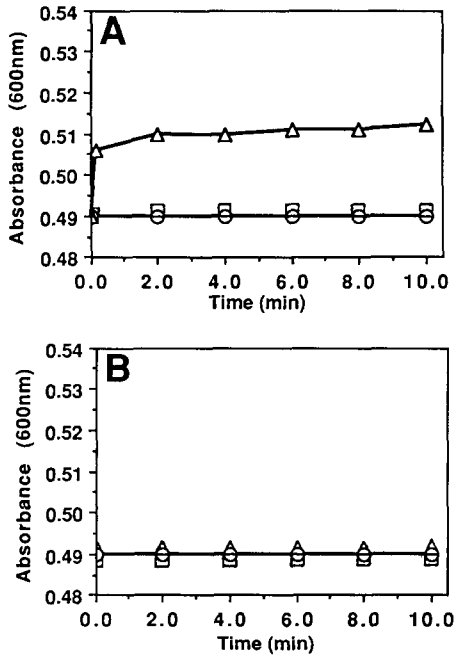


Fig. 6. Aggregation of bacterial cells in the presence of catechins. Bacterial cell suspension ($A_{600} = 0.5$, 686 μl) was mixed with catechin (62.5 mM/14 μl), and the turbidity recorded at A_{600}. (A) *S. aureus*. ○, without catechin; △, 1.25 mM EGCg; □, 1.25 mM EC. (B) *E. coli*. ○, without catechin; △, 1.25 mM EGCg; □, 1.25 mM EC.

It is possible, therefore, that EGCg interacts with the membrane of Gram-positive bacteria more readily than that of Gram-negative bacteria. We tested this possibility by determining the adsorption of EGCg onto the intact bacterial cell. The results showed that *S. aureus* absorbed about 2.5 times more EGCg than *E. coli* per unit weight of bacterial cell (data not shown). Therefore, it is likely that the lower EGCg susceptibility of *E. coli*, compared with *S. aureus*, is due to low accessibility and less binding, in addition to the barrier function of the outer membrane.

Discussion

Tea and coffee, the most common drinks served nearly every day to the vast majority of people in the world. Tea, more precisely the aqueous extract of tea leaves, had been shown to have antibacterial [1–3], antiviral [4–6] and protein denaturating [10–12] activities in vitro. These biological activities of tea extracts are attributable to the presence of polyphenol compounds [7], collectively termed catechins. Catechins inhibit the growth of bacterial cells and the bactericidal activity is much higher against Gram-positive than Gram-negative bacteria [7–9].

We have reported in this paper the mode of action of bactericidal catechins in vitro. We found that bactericidal catechin (EGCg) caused rapid leakage of small molecules entrapped in the intraliposomal space (Fig. 2). CF-leakage was undetectably low in the presence of non-bactericidal catechin, EC. Leakage of CF was significantly lowered when the liposome membranes contained negatively charged lipids such as PC and DCP. These results suggest that the low catechin susceptibility of Gram-negative bacteria is attributable, at least in part, to the presence of a strong negative charge on the cell surface, though the tight penetration barrier of the outer membrane against hydrophobic and large hydrophilic compounds might also contribute to a certain extent.

However, the question of how bactericidal catechins damage the bacterial cell membrane still remains unanswered. Earlier reports showed that the catechin was incorporated into the plasma membrane of rat hepatocyte [18], and caused reduced flux of thiourea and cycloleucine in the mouse ascite tumor cell [17]. This study revealed that bactericidal catechin caused leakage of intramembranous materials (Fig. 3), and aggregation of the liposomes (Figs. 5 and 6). Therefore, it is likely that the catechins interact with the membrane and perturb the lipid bilayers possibly by the catechin directly penetrating the lipid bilayer and disrupting the barrier function. This possibility was supported by the observation that the catechin caused CF-release and the liposome aggregation (Figs. 3, 5 and 6). Direct incorporation of catechin into the membrane was also demonstrated using radiolabeled catechin [18]. It was suggested that catechins may change the membrane fluidity [18]. An alternative interpretation of the data would be the possibility that catechins caused membranes fusion. The data showing that catechins caused leakage of intraliposomally entrapped small molecules and strong aggregation of liposomes and bacterial cells point to the likely possibility of membrane fusion. It is well established that membrane fusion by polyethylene glycol, calcium ion or virions, has consistently resulted in the leakage of intramembranous materials and aggregation (see Ref. 22 and 23 for reviews). However, the data so far available neither prove nor rule out this possibility. This question has to be assessed in the future.

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