Original Article

Epigallocatechin-3-Gallate Has an Anti-Platelet Effect in a Cyclic AMP-Dependent Manner

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Aim: In this study, we investigated the effect of (−)-epigallocatechin-3-gallate (EGCG) on cyclic nucleotide production and vasodilator-stimulated phosphoprotein (VASP) phosphorylation in collagen (10 μg/mL)-stimulated platelet aggregation.

Methods: Washed platelets (10⁸/mL) from Sprague-Dawley rats (6-7 weeks old, male) were preincubated for 3 min at 37 °C in the presence of 2 mM exogenous CaCl₂ with or without EGCG or other materials, stimulated with collagen (10 μg/mL) for 5 min, and then used for the determination of intracellular cytosolic Ca²⁺ ([Ca²⁺]ᵢ), thromboxane A₂ (TXA₂), adenosine 3',5'-cyclic monophosphate (cAMP), guanosine 3',5'-cyclic monophosphate (cGMP), and VASP phosphorylation.

Results: EGCG dose-dependently inhibited collagen-induced platelet aggregation by inhibiting both [Ca²⁺]ᵢ mobilization and TXA₂ production. Of two aggregation-inhibiting molecules, cAMP and cGMP, EGCG significantly increased intracellular levels of cAMP, but not cGMP. EGCG-elevated cAMP level was decreased by SQ22536, an adenylyl cyclase inhibitor, but not by etazolate, a cAMP-specific phosphodiesterase inhibitor. In addition, EGCG elevated the phosphorylation of VASP-Ser¹⁵⁷, a cAMP-dependent protein kinase (A-kinase) substrate, but not the phosphorylation of VASP-Ser²³⁹, a cGMP-dependent protein kinase substrate, in intact platelets and collagen-induced platelets, and VASP-Ser¹⁵⁷ phosphorylation by EGCG was inhibited by both an adenylyl cyclase inhibitor SQ22536 and an A-kinase inhibitor Rp-8-Br-cAMPS. We have demonstrated that EGCG increases cAMP via adenylyl cyclase activation and subsequently phosphorylates VASP-Ser¹⁵⁷ through A-kinase activation to inhibit [Ca²⁺]ᵢ mobilization and TXA₂ production on collagen-induced platelet aggregation.

Conclusions: These results strongly indicate that EGCG is a beneficial compound elevating cAMP level in collagen-platelet interaction, which may result in the prevention of platelet aggregation-mediated thrombotic diseases.


Key words: (−)-Epigallocatechin-3-gallate, Adenosine 3',5'-cyclic monophosphate, Intracellular cytosolic Ca²⁺, Thromboxane A₂, Vasodilator-stimulated phosphoprotein-Ser¹⁵⁷ phosphorylation

Introduction

Platelet aggregation is absolutely essential for the formation of a haemostatic plug when normal blood vessels are injured; however, the interactions between platelets and collagen can also cause circulatory disorders, such as thrombosis, atherosclerosis, and myocardial infarction¹. Accordingly, inhibition of the plate-
let-collagen interaction might be a promising approach for the prevention of thrombosis. An important role in the mechanism by which collagen induces platelet aggregation is played by thromboxane A2 (TXA2) formation, which also contributes to an increase in cytosolic free Ca2+ ([Ca2+]i). An increase in [Ca2+]i activates both the Ca2+/calmodulin-dependent phosphorylation of myosin light chain and the diacylglycerol (DG)-dependent phosphorylation of pleckstrin to induce platelet aggregation. In addition, DG can be hydrolyzed by DG lipase to produce arachidonic acid (20:4), a precursor of TXA2, which is a potent platelet aggregation agent generated from 20:4 liberated when phosphatidylinositol 4, 5-bisphosphate (PIP2) is broken down by phospholipase C (PLC) in collagen-, thrombin-, and ADP-activated platelets. Verapamil and theophylline have anti-platelet functions that elevate the adenosine 3′, 5′-cyclic monophosphate (cAMP) level, and then decrease [Ca2+]i, an essential factor for platelet aggregation. Vasodilators (such as molsidomine and nitroprusside) and guanosine 3′, 5′-cyclic monophosphate (cGMP) phosphodiesterase (PDE) inhibitors (such as zaprinast and 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-8-Br-cAMPS), 9-(tetrahydro-2′-furyl)adenine (SQ22536), 1-ethyl-4-[(1-methylethyliden)cyclohexyl]hydrazino] 1H-pyrazolo[3,4-b]pyridine-5-carboxylic acid (Etazolate), (−)-epigallocatechin-3-gallate (EGCG), and other reagents were obtained from Sigma Chemical Corporation (St. Louis, MO, USA). cAMP, cGMP, and TXB2 enzyme immunoassay (EIA) kits were purchased from GE Healthcare (Buckinghamshire, UK).

Materials and Methods

Collagen was obtained from the Chrono-Log Corporation (Havertown, PA, USA). Fura 2-AM, 8-bromoadenosine-3′, 5′-cyclic monophosphorothioate, Rp-isomer (Rp-8-Br-cAMPS), 9-(tetrahydro-2′-furyl)adenine (SQ22536), 1-ethyl-4-[(1-methylethylidene)hydrazino] 1H-pyrazolo[3,4-b]pyridine-5-carboxylic acid (Etazolate), (−)-epigallocatechin-3-gallate (EGCG), and other reagents were obtained from Sigma Chemical Corporation (St. Louis, MO, USA). cAMP, cGMP, and TXB2 enzyme immunoassay (EIA) kits were purchased from GE Healthcare (Buckinghamshire, UK).

Preparation of Washed Rat Platelets

Blood was collected from Sprague-Dawley rats (6-7 weeks old, male), and anti-coagulated with ACD solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose). Platelet-rich plasma was centrifuged at 125×g for 10 min to remove red blood cells, and the platelets were washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO3, 0.36 mM NaH2PO4, 5.5 mM glucose, and 1 mM EDTA, pH 7.4). The washed platelets were then resuspended in suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO3, 0.36 mM NaH2PO4, 0.49 mM MgCl2, 5.5 mM glucose, 0.25% gelatin, pH 7.4) to a final concentration of 5×10^9/mL. All of the above procedures were carried out at 25°C to avoid platelet aggregation on cooling. The ethics committee
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sensitive, so the tube containing the platelet-rich plasma was covered with aluminum foil during loading. The fura 2-loaded washed platelets were prepared using the procedure described above and platelets 10^{8}/mL were preincubated for 3 min at 37°C with various concentrations of EGCG in the presence of 2 mM CaCl₂ and then stimulated with collagen (10 μg/mL) for 5 min for evaluation of [Ca^{2+}]_{i}. Fura 2 fluorescence was measured with a spectrofluorimeter (SFM 25; Bio-Tech Instrument, Italy) with an excitation wavelength that was changed every 0.5 sec from 340 to 380 nm; the emission wavelength was set at 510 nm. The [Ca^{2+}]_{i} values were calculated using the method of Schaeffer^{22}).

Measurement of TXB₂

Washed platelets (10^{8}/mL) were preincubated with or without EGCG for 3 min in the presence of 2 mM CaCl₂ for animal experiments of Inje University (Gimhae, Gyeongnam, Korea) approved these animal experiments.

Measurement of Platelet Aggregation

Washed platelets (10^{8}/mL) were preincubated for 3 min at 37°C in the presence of 2 mM exogenous CaCl₂ with or without EGCG and then stimulated with collagen (10 μg/mL) for 5 min. Aggregation was monitored using an aggregometer (Chrono-Log, Corp.) at a constant stirring speed of 1,000 rpm. Each aggregation rate was evaluated as an increase in light transmission. Suspension buffer was used as a reference. EGCG was dissolved in distilled water.

Determination of [Ca^{2+}]_{i}

Platelet-rich plasma was incubated with 5 μM fura 2-AM at 37°C for 60 min. Fura 2-AM is light-sensitive, so the tube containing the platelet-rich plasma was covered with aluminum foil during loading. The fura 2-loaded washed platelets were prepared using the procedure described above and platelets 10^{8}/mL were preincubated for 3 min at 37°C with various concentrations of EGCG in the presence of 2 mM CaCl₂ and then stimulated with collagen (10 μg/mL) for 5 min for evaluation of [Ca^{2+}]_{i}. Fura 2 fluorescence was measured with a spectrofluorimeter (SFM 25; Bio-Tech Instrument, Italy) with an excitation wavelength that was changed every 0.5 sec from 340 to 380 nm; the emission wavelength was set at 510 nm. The [Ca^{2+}]_{i} values were calculated using the method of Schaeffer^{22}).

Measurement of TXB₂

Washed platelets (10^{8}/mL) were preincubated with or without EGCG for 3 min in the presence of 2

Fig. 1. Effects of EGCG on collagen-stimulated platelet aggregation. (A) Chemical structure of EGCG from green tea leaves. (B) The concentration threshold of collagen on platelet aggregation. Washed platelets (10^{8}/mL) were stimulated with various doses of collagen for 5 min in the presence of 2 mM CaCl₂ at 37°C. (C) Effects of EGCG pretreatment on collagen-stimulated platelet aggregation. Washed platelets (10^{8}/mL) were preincubated with various concentrations of EGCG (1 to 50 μM) in the presence of 2 mM CaCl₂ for 3 min at 37°C, and then stimulated with collagen (10 μg/mL) for 5 min. Platelet aggregation (%) was recorded as an increase in light transmission. Inhibition by EGCG was recorded as a percentage of the collagen-induced aggregation rate. Data are expressed as the means ± S.E.M. (n=4). *p<0.05. **p<0.001.
mM CaCl₂, and activated for 5 min with collagen (10 μg/mL). The reactions were terminated by the addition of ice-cold EDTA (5 mM) and indomethacin (0.2 mM). The amount of TXB₂, a stable metabolite of TXA₂, was determined with Synergy HT Multi-Model Microplate Reader (BioTek Instruments, Winoosku, VT, USA) using a TXB₂ EIA kit.

Measurement of cAMP and cGMP
Washed platelets (10⁸/mL) were preincubated for 3 min at 37°C with various concentrations of EGCG or other agonists in the presence of 2 mM CaCl₂, and then stimulated with collagen (10 μg/mL) for 5 min for platelet aggregation. The aggregation was terminated by the addition of 80% ice-cold ethanol. cAMP and cGMP were measured Synergy HT Multi-Model Microplate Reader (BioTek Instruments) using cAMP and cGMP EIA kits.

Western Blot for Analysis of VASP Phosphorylation
Various volumes of platelet lysates containing the same protein (15 μg) were used for analysis. Protein concentrations were measured using a BCA protein assay kit (Pierce Biotechnology, USA). An 8-10% SDS-PAGE was used for electrophoresis and a PVDF membrane was used for protein transfer from the gel. The dilutions for anti-VASP, anti-phosphor-VASP (Ser₁₅₇), and anti-rabbit IgG-HRP were 1:1000, 1:1000, 1:1000, and 1:10000, respectively. The membranes were visualized using enhanced chemiluminescence (ECL). Blots were analyzed using Quantity One, Ver. 4.5 (Bio-Rad, Hercules, CA, USA).

Statistical Analysis
The experimental results are expressed as the means ± S.E.M. and are accompanied by the number of observations. Data were assessed by analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared by the Newman-Keuls method. P < 0.05 was considered significant.

Results
Effects of EGCG on Collagen-Induced Platelet Aggregation
The concentration of collagen-induced maximal platelet aggregation was approximately 10 μg/mL (Fig. 1B)²³; therefore, 10 μg collagen/mL was used as the platelet agonist in this study. When washed platelets (10⁸/mL) were activated with 10 μg collagen/mL in the presence of 2 mM CaCl₂, the aggregation rate was increased up to 81.5 ± 2.4%; however, various concentrations of EGCG (1 to 50 μM) significantly reduced collagen-stimulated platelet aggregation in a dose-dependent manner (Fig. 1C).

Effects of EGCG on Regulation of Aggregation-Inducing Molecules, [Ca²⁺] and TXA₂
As shown in Fig. 2A, when washed platelets (10⁸/mL) were stimulated by collagen (10 μg/mL), the level of [Ca²⁺] increased from 48.6 ± 9.2, the basal level, to 90.2 ± 8.3 nM; however, this was significantly
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reduced by various concentrations (1 to 50 μM) of EGCG in a dose-dependent manner (83.5% inhibition at 50 μM). We next determined whether EGCG blocks the production of TXA₂ by collagen stimulation. The TXA₂ (determined as TXB₂) level in intact platelets was 4.0 ± 0.1 ng/10⁸ platelets, and this was markedly increased to 356.1 ± 46.9 ng/10⁸ platelets in collagen-stimulated platelets (Fig. 2B); however, EGCG potently reduced the production of TXA₂ to 37.3 ± 0.4 ng/10⁸ platelets (89.5% inhibition at 50 μM). Because collagen-produced TXA₂ resulted from Ca²⁺-dependent PLC activation, it is suggested that the inhibition of TXA₂ production (Fig. 2B) by EGCG might be associated with the inhibition of collagen-elevated [Ca²⁺]i (Fig. 2A). Intracellular cAMP or cGMP reduce platelet agonist-elevated [Ca²⁺]i and TXA₂ to inhibit platelet aggregation 24; therefore, we next investigated whether EGCG up-regulates the cellular level of cAMP or cGMP.

Effects of EGCG on Production of cGMP and cAMP, Aggregation-Inhibiting Molecules

Collagen decreased intracellular cGMP from 1.40 ± 0.15 pmoL/10⁸ platelets (basal level) to 1.21 ± 0.15 pmoL/10⁸ platelets in washed platelets (Table 1). When the platelets were incubated in the presence of both EGCG and collagen, cGMP by EGCG did not increase in a dose-dependent manner (Fig. 3A). The cGMP level (1.43 ± 0.04 pmoL/10⁸ platelets) in the presence of both EGCG (50 μM) and collagen (10 μg/mL) increased to 18.2 % as compared with that (1.21 ± 0.15 pmoL/10⁸ platelets) in the presence of collagen (10 μg/mL) alone (Table 1).

Collagen decreased intracellular cAMP from 3.89 ± 0.18 pmoL/10⁸ platelets (basal level) to 3.44 ± 0.43 pmoL/10⁸ platelets in washed platelets (Table 1); however, when the platelets were incubated in the presence of both EGCG and collagen, cAMP by EGCG increased in a dose-dependent manner (Fig. 3B). The cAMP level (6.60 ± 0.04 pmoL/10⁸ platelets) in the

Table 1. Changes of cAMP and cGMP

<table>
<thead>
<tr>
<th></th>
<th>cAMP (pmoL/10⁸ platelets)</th>
<th>Change (%)</th>
<th>cGMP (pmoL/10⁸ platelets)</th>
<th>Change (%)</th>
<th>cAMP/cGMP Ratio</th>
<th>Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base</td>
<td>3.89 ± 0.18</td>
<td>0</td>
<td>1.40 ± 0.15</td>
<td>0</td>
<td>2.8</td>
<td>0</td>
</tr>
<tr>
<td>EGCG (50 μM)</td>
<td>5.31 ± 0.80</td>
<td>+36.5</td>
<td>1.30 ± 0.10</td>
<td>−7.1</td>
<td>4.1</td>
<td>46.4</td>
</tr>
<tr>
<td>Collagen (10 μg/mL)</td>
<td>3.44 ± 0.43</td>
<td>0</td>
<td>1.21 ± 0.15</td>
<td>0</td>
<td>2.8</td>
<td>0</td>
</tr>
<tr>
<td>Collagen + EGCG</td>
<td>6.60 ± 0.04</td>
<td>+91.9</td>
<td>1.43 ± 0.04</td>
<td>+18.2</td>
<td>4.6</td>
<td>64.3</td>
</tr>
</tbody>
</table>

Data were from Fig. 3. 1), 2), and 5) changes to base; 3), 4), and 6) changes to collagen.

Fig. 3. Effects of EGCG on cAMP and cGMP production in resting and collagen-stimulated platelets. (A) Effects of EGCG on cGMP production in resting or collagen-stimulated platelets. (B) Effects of EGCG on cAMP production in resting or collagen-stimulated platelets. Washed platelets (10⁸/mL) were preincubated with or without EGCG for 3 min in the presence of 2 mM CaCl₂ and then stimulated with collagen (10 μg/mL) for 5 min at 37°C. The reactions were terminated by adding 80% ice-cold ethanol. cGMP contents were measured using EIA kits. Data are expressed as the means ± S.E.M. (n=4). *p<0.05. **p<0.001.
presence of both EGCG (50 μM) and collagen (10 μg/mL) increased to 91.9% as compared with that (3.44 ± 0.43 pmoL/10⁸ platelets) in the presence of collagen (10 μg/mL) alone (Table 1).

**Effects of EGCG on Phosphorylation at Ser¹⁵⁷ or Ser²³⁹ of VASP**

In intact platelets, basal 46 kDa dephosphoprotein of VASP only was observed and was shifted to 50 kDa phosphoprotein of VASP in collagen-induced platelet aggregation (Fig. 4A lane 1, 2). Collagen (10 μg/mL) increased p-VASP (Ser¹⁵⁷ and Ser²³⁹) at 50 kDa phosphoprotein of VASP (Fig. 4A lane 2). It is known that thrombin and collagen, agonists of platelets, are involved in a feedback inhibition by elevating p-VASP (Ser157 and Ser 239)²⁵). EGCG itself stimulated the shift from 46 kDa dephosphoprotein of VASP to 50 kDa phosphoprotein of VASP, and markedly increased the p-VASP (Ser¹⁵⁷) only of VASP-50 kDa, which is observed in collagen-induced platelet aggre-
gation. The band density (21971 density/mm²) of p-VASP (Ser^{157}) by both EGCG and collagen was increased to 16.6% as compared with that (18841 density/mm²) by collagen (Fig. 4B, D). This suggests that EGCG strongly phosphorylates VASP (Ser^{157}), indicating the relation to A-kinase activation by EGCG-elevated cAMP. The band density (18050 density/mm²) of p-VASP (Ser^{239}) by both EGCG and collagen was increased to 5.5% as compared with that (17188 density/mm²) by collagen (Fig. 4C, D). The phosphorylation of VASP (Ser^{239}) by EGCG is much slighter than p-VASP (Ser^{157}) (Fig. 4A lane 3, 4, C and D). This suggests that EGCG slightly phosphorylates VASP (Ser^{157}), indicating a relation to G-kinase activation of cGMP elevated slightly by EGCG.

**Effects of EGCG on cAMP Production and VASP Phosphorylation in the Presence of Adenylate Cyclase Inhibitor, SQ22536**

The cAMP level is regulated by adenylate cyclase, cAMP-specific phosphodiesterase (PDE4), cGMP-stimulated phosphodiesterase (PDE2) and cGMP-inhibited phosphodiesterase (PDE3) in platelets. Because EGCG hardly affected cGMP production in both resting and collagen-stimulated platelets (Fig. 3A), it is inferred that EGCG-elevated cAMP was not regulated by the inhibition of PDE2 hydrolyzing both cAMP and cGMP, and PDE3 hydrolyzing cAMP rather than cGMP. Accordingly, we set out to investigate in this study whether the EGCG-mediated cAMP level was regulated by enzymes of adenylate cyclase or PDE4. For the first time, we used SQ22536, an adenylate cyclase inhibitor, in this study whether the EGCG-mediated cAMP rather than cGMP . Accordingly, we set out to investigate whether the EGCG-mediated cAMP level was regulated by enzymes of adenylate cyclase or PDE4. For the first time, we used SQ22536, an adenylate cyclase inhibitor, in this study whether the EGCG-mediated cAMP rather than cGMP was regulated by the inhibition of PDE2 hydrolyzing both cAMP and cGMP, and PDE3 hydrolyzing cAMP rather than cGMP. Accordingly, we set out to investigate in this study whether the EGCG-mediated cAMP level was regulated by enzymes of adenylate cyclase or PDE4. For the first time, we used SQ22536, an adenylate cyclase inhibitor, in this study whether the EGCG-mediated cAMP rather than cGMP.

**Fig. 5.** Effects of EGCG on cAMP production and VASP phosphorylation in resting and collagen-stimulated platelets. (A) Effects of EGCG with SQ22536, an adenylate cyclase inhibitor, on cAMP production in collagen-stimulated platelets. Washed platelets (10^6/mL) were preincubated with or without EGCG or SQ22536 for 3 min in the presence of 2 mM CaCl2 and then stimulated with collagen (10 μg/mL) for 5 min at 37°C in an aggregometer. The reactions were terminated by adding 80% ice-cold ethanol. cAMP contents were measured using EIA kits. Data are expressed as the means ± S.E.M. (n = 4). *p < 0.05. **p < 0.001. (B) Effects of EGCG with SQ22536, adenylate cyclase inhibitor, on VASP (Ser^{157}) phosphorylation [p-VASP (Ser^{157})]. Lane 1, collagen (10 μg/mL); lane 2, EGCG (50 μM); lane 3, collagen (10 μg/mL) + EGCG (50 μM); lane 4, SQ22536 (10 μM); lane 5, collagen (10 μg/mL) + EGCG (50 μM) + SQ22536 (10 μM); lane 6, forskolin (10 μM). Proteins were extracted, separated by SDS-PAGE transferred to PVDF and immunoblotted with the indicated corresponding antibodies, anti-phosphor VASP or Ser^{157}. Blots were visualized by the ECL plus kit (Amersham) and immunoblot panels are representative of 3-4 similar experiments. Blots were analyzed using Quantity One, Ver. 4.5 program (Bio-rad), and units are expressed as intensity/mm².

**Effects of EGCG on Platelet Aggregation and VASP Phosphorylation in the Presence of cAMP-Dependent Protein Kinase (A-kinase) Inhibitor Rp-8-Br-cAMPS**

The inhibitory action mode of platelet aggrega-
**Table 2.** Changes of p-VASP (Ser^{157}) by SQ22536

<table>
<thead>
<tr>
<th></th>
<th>Blot (intensity/mm^2)</th>
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<tbody>
<tr>
<td></td>
<td>Collagen (10 µg/mL)</td>
</tr>
<tr>
<td>VASP 50 kDa</td>
<td>19403 ± 2941</td>
</tr>
<tr>
<td>46 kDa</td>
<td>14560 ± 1471</td>
</tr>
<tr>
<td>p-VASP (Ser^{157})</td>
<td>50 kDa</td>
</tr>
<tr>
<td>VASP (50 kDa)</td>
<td>1.22</td>
</tr>
</tbody>
</table>

Data were from Fig. 5. *p < 0.05 vs collagen. **p < 0.05 vs collagen + EGCG.

**Table 3.** Changes (%) of p-VASP (Ser^{157}) by EGCG or SQ22536

<table>
<thead>
<tr>
<th>Collagen</th>
<th>Collagen + EGCG</th>
<th>Collagen + EGCG + SQ22536</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>+ 6.3</td>
<td>- 22.7</td>
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</table>

Data are calculated from Table 2. 1) change to collagen; 2) change to collagen plus EGCG.

The activation of A-kinase which phosphorylates VASP (Ser^{157}) in platelet aggregation; therefore, because the inhibition of A-kinase activation and VASP (Ser^{157}) phosphorylation induced platelet aggregation, we investigated whether EGCG affects collagen-stimulated platelet aggregation and VASP (Ser^{157}) phosphorylation in the presence of A-kinase inhibitor, Rp-8-Br-cAMPS.

As shown in Fig. 6A, in the presence of both Rp-8-Br-cAMPS and EGCG, the degree of collagen-induced platelet aggregation (58.0 ± 2.0%) was higher than that (18.0 ± 3.0%) in the presence of EGCG alone. The band density (Fig. 6B lane 2, Table 4) of p-VASP (Ser^{157}) phosphorylated by both collagen and EGCG in the presence of Rp-8-Br-cAMPS was decreased to 76.1% as compared with that (Fig. 6B lane 1, Table 4) in the absence of Rp-8-Br-cAMPS. These results reflect that the inhibitory effect of EGCG on collagen-induced platelet aggregation is involved in the phosphorylation of VASP (Ser^{157}) by the cAMP/A-kinase pathway.

**Effects of EGCG on cAMP Production in the Presence of PDE4 Inhibitor Etazolate**

As shown in Fig. 7, when washed platelets were stimulated by collagen in the presence of etazolate alone (20 µM), the cAMP level was increased significantly from 3.5 ± 0.3 pmoL/10^8 platelets to 5.3 ± 0.5 pmoL/10^8 platelets. On the other hand, when washed platelets were stimulated by collagen in the presence of both etazolate and EGCG, the cAMP level was increased up to 6.2 ± 0.6 pmoL/10^8 platelets (Fig. 7). This cAMP level (6.2 ± 0.6 pmoL/10^8 platelets), however, was not synergistically increased as compared with that (6.6 ± 0.1 pmoL/10^8 platelets) increased by EGCG in collagen-stimulated platelet aggregation. These results suggest that EGCG-activated cAMP enhancement (Fig. 3B, 7) is not mediated by the inhibition of cAMP-specific phosphodiesterase, PDE4.

**Discussion**

Catechins that have an anti-inflammatory effect are known to have anti-platelet function by inhibiting the liberation of TXA_2 precursor 20:4 out of platelet membrane phospholipids and TXA_2 synthase activity in collagen-activated platelets

EGCG has been reported to inhibit collagen-activated Ca^{2+}-dependent PLC-γ2 and TXA_2 production. Since EGCG inhibited [Ca^{2+}]_i mobilization (Fig. 2A) and TXA_2 production (Fig. 2B), but increased cAMP production (Fig. 3B, Table 1), as in previous reports, this suggested that EGCG-activated Ca^{2+}-antagonistic cAMP (Fig. 3B) may be involved in the inhibition of 20:4 liberation and TXA_2 production by suppressing [Ca^{2+}]_i mobilization and Ca^{2+}-dependent PLC-γ2 activity. TXA_2 is produced via cyclooxygenase-1 (COX-1) and the TXA_2 synthase (TXAS) pathway from 20:4. In our other experiments, EGCG inhibited microsomal COX-1 activity to 91% as compared with that (1.4 nmoL/protein-mg/min) of the control, and inhibited microsomal TXAS activity to 20% as compared with the control (0.17 nmoL/protein-mg/min) (data not shown). It is thought that EGCG has multiple actions that reduce [Ca^{2+}]_i mobilization and TXA_2 production-associated enzyme (COX-1, TXAS) activities to inhibit TXA_2 production, and 20:4 is metabolized to prostaglandin E_2, an inflammatory mediator.
by cyclooxygenase-2 (COX-2). Because it was observed that EGCG inhibits TXA₂ production (Fig. 2B) and microsomal COX-1 (data not shown), it is clear that EGCG has an anti-inflammatory effect by inhibiting COX-2 activity, in the same way as EGCG has an anti-prostate carcinogenic effect by inhibiting COX-2 activity. Because both platelet aggregation and inflammation are the cause of atherosclerosis, it is thought that EGCG could contribute to the treatment of cardiovascular disease.

A-kinase and G-kinase are key modulators controlling platelet aggregation (Table 1). EGCG significantly increased cAMP rather than cGMP in intact and collagen-induced platelet aggregation (Table 1). EGCG itself increased up to 46.4% the ratio of cAMP to cGMP from 2.8, the basal ratio, to 4.1 in unstimulated platelets (Table 1). The ratio of cAMP to cGMP was increased up to 64.3% in the presence of both collagen and EGCG as compared with that (2.8) in the absence of EGCG (Table 1). This suggests that EGCG directly produced cAMP and used it to decrease [Ca²⁺]ᵢ, an essential molecule for TXA₂ production. cAMP and cGMP are involved in the inhibition of platelet aggregation by phosphorylating IP₃ receptor or VASP via the A-kinase or G-kinase pathway. IP₃ receptor phosphorylation is involved in the inhibition of [Ca²⁺]ᵢ mobilization and VASP phosphorylation is known to regulate VASP affinity for platelet filamentous actin. In the present study, it is unknown whether the inhibitory effect of [Ca²⁺]ᵢ by EGCG is due to the phosphorylation of IP₃ receptor by cAMP or cGMP. In intact and collagen-activated platelets, EGCG phosphorylated A-kinase substrate VASP (Ser 157) markedly, but did not phosphory-

**Fig. 6.** Effect of EGCG on collagen-induced platelet aggregation and VASP (Ser 157) phosphorylation in the presence of A-kinase inhibitor, Rp-8-Br-cAMPS. (A) Effects of EGCG in the presence of Rp-8-Br-cAMPS on collagen-stimulated platelets. Washed platelets (10⁵/mL) were preincubated with EGCG (50 μM) with Rp-8-Br-cAMPS (20 μM), a A-kinase inhibitor, in the presence of 2 mM CaCl₂ for 3 min at 37°C, and then stimulated with collagen (10 μg/mL) for 5 min. Platelet aggregation (%) was recorded as an increase in light transmission. Data are expressed as the means ± S.E.M. (n=4). **p < 0.001. (B) Effects of EGCG in the presence of Rp-8-Br-cAMPS on VASP (Ser 157). Lane 1, collagen + EGCG; lane 2, collagen + EGCG + Rp-8-Br-cAMPS. Collagen-induced platelet aggregation was terminated by adding an equal volume of lysis buffer. Proteins were extracted, separated by SDS-PAGE (8-10%), transferred to PVDF and immunoblotted with the indicated corresponding antibodies, anti-phosphor VASP , or Ser157. Blots were visualized by the ECL plus kit (Amersham) and immunoblot panels are representative of 3-4 similar experiments. Blots were analyzed using Quantity One, Ver. 4.5 program (Bio-rad), and units are expressed as intensity/mm².

**Table 4.** Changes (%) of p-VASP (Ser 157) by EGCG or Rp-8-Br-cAMPS

<table>
<thead>
<tr>
<th>Band density of p-VASP (Ser 157) (Intensity/mm²)</th>
<th>Inhibition of p-VASP (Ser 157) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen + EGCG</td>
<td>31800</td>
</tr>
<tr>
<td>Collagen + EGCG + Rp-8-Br-cAMPS</td>
<td>7600</td>
</tr>
</tbody>
</table>

Data were calculated from Fig. 6B.
late G-kinase substrate VASP (Ser^{239}) (Fig. 4A). These results are in accord with the result that EGCG markedly enhanced cAMP rather than cGMP in intact and collagen-stimulated platelets (Table 1). Eigenthaler et al.\(^{33}\) reported that a small elevation in cAMP is enough to activate most A-kinase, whereas even several fold elevation of the cGMP level may stimulate only a small fraction of total G-kinase. This previous report\(^{33}\) reflects that even a little cAMP is enough to phosphorylate VASP (Ser^{239}), an A-kinase substrate, and much cGMP is required to phosphorylate VASP (Ser^{239}), a G-kinase substrate. Accordingly, even though EGCG increased cGMP to 18.2% in collagen-stimulated platelets (Table 1), even though EGCG increased cGMP to 18.2% in collagen-stimulated platelets; however, EGCG markedly increased cAMP level, and hardly increased the cGMP level (Table 1). In particular, since EGCG did not increase but decreased cGMP production in intact platelets (Table 1), even though the cGMP level (1.43 ± 0.04 pmoL/10^8 platelets) was increased to 18.2% in the presence of both collagen and EGCG as compared with that (1.21 ± 0.15 pmoL/10^8 platelets) in the absence of EGCG (Table 1), it is thought that EGCG-elevated cAMP (Fig. 3B) is not due to the regulation of cGMP-stimulated PDE2, cGMP-inhibited PDE3, and cGMP-binding-cGMP-specific PDE5. EGCG increased cGMP to about 0.22 pmoL/10^8 platelets as compared with that (1.21 ± 0.15 pmoL/10^8 platelets) in the presence of collagen (10 μg/mL) (Table 1, Fig. 3A). This increased cGMP level (0.22 pmoL/10^8 platelets) is not sufficient to inhibit PDE3 to increase cAMP, since it is known that Ki for cGMP inhibition of cAMP hydrolysis by PDE3 is 0.06 μM (60 pmoL/mL)\(^{35}\); therefore, EGCG-elevated cAMP (Table 1, Fig. 3B) is not due to the inhibition of PDE3.

The next question therefore concerns the mechanism by which EGCG up-regulated the intracellular cAMP level. To solve this question, we used SQ22536 as an adenylate cyclase inhibitor and etazolate as a PDE4 inhibitor. The EGCG-elevated cAMP level was decreased by SQ22536 (Fig. 5A), which results from inhibition of adenylate cyclase by SQ22536. Because SQ22536 decreased cAMP production, the phosphorylation of VASP (Ser^{157}) in the presence of EGCG was inhibited by SQ22536 (Fig. 5B). These results suggest that EGCG produced cAMP from ATP by activating adenylate cyclase, and phosphorylated VASP (Ser^{157}) via both cAMP production and A-kinase activation. Sudo et al.\(^{34}\), using cilostazole, reported a similar result to our finding, and suggested that its antiplatelet effect is mediated through an increase of the cAMP level, A-kinase activity, and VASP phosphorylation. The levels of intracellular cAMP and cGMP are regulated by the balance between cyclic nucleotide-producing enzymes, adenylate/guanylate cyclases, and hydrolyzing enzymes, cAMP/cGMP PDEs. If EGCG inhibited the activity of PDE2 to produce cAMP and cGMP in intact and collagen-stimulated platelets, because PDE2 hydrolyzes both cAMP and cGMP,\(^{26}\) EGCG would increase the level of cAMP and cGMP in intact and collagen-stimulated platelets; however, EGCG markedly increased the cAMP level only, and hardly increased the cGMP level (Table 1). In particular, since EGCG did not increase but decreased cGMP production in intact platelets (Table 1), even though the cGMP level (1.43 ± 0.04 pmoL/10^8 platelets) was increased to 18.2% in the presence of both collagen and EGCG as compared with that (1.21 ± 0.15 pmoL/10^8 platelets) in the absence of EGCG (Table 1), it is thought that EGCG-elevated cAMP (Fig. 3B) is not due to the regulation of cGMP-stimulated PDE2, cGMP-inhibited PDE3, and cGMP-binding-cGMP-specific PDE5. EGCG increased cGMP to about 0.22 pmoL/10^8 platelets as compared with that (1.21 ± 0.15 pmoL/10^8 platelets) in the presence of collagen (10 μg/mL) (Table 1, Fig. 3A). This increased cGMP level (0.22 pmoL/10^8 platelets) is not sufficient to inhibit PDE3 to increase cAMP, since it is known that Ki for cGMP inhibition of cAMP hydrolysis by PDE3 is 0.06 μM (60 pmoL/mL)\(^{35}\); therefore, EGCG-elevated cAMP (Table 1, Fig. 3B) is not due to the inhibition of PDE3.

![Fig. 7. Effects of EGCG with etazolate, cAMP-specific phosphodiesterase inhibitor, on cAMP production in collagen-stimulated platelets.](image-url)
ently increased to 657, 4300, and 4410 pmol EGCG/mL, in subjects who received 3, 5, and 7 capsules, respectively (corresponding to 225, 375, and 525 mg EGCG, respectively). EGCG levels detected in plasma corresponded to 0.2-2.0% of the ingested amount. In the present study, although it is unknown whether 46-460 μg, corresponding to 0.2-2.0% of EGCG (MW. 458.37) 50 μM (23 mg) having anti-platelet activities, has clinic anti-platelet activity in vivo, which should be studied in the future, and if it has a dietary effect, it could be used to evaluate the anti-platelet effects of EGCG.

In conclusion, the most important result of this study is that EGCG significantly inhibits the level of [Ca2+]i and TXA2, platelet-aggregating molecules. The inhibition of [Ca2+]i by EGCG depends on the up-regulation of Ca2+-antagonistic intracellular cAMP level through the activation of adenylyl cyclase. Furthermore, our findings showed that EGCG-elevated cAMP phosphorylates VASP (Ser157) through A-kinase activation to inhibit collagen-induced platelet aggregation. Therefore, these results suggest that EGCG is a physiologically effective negative feedback regulator during platelet aggregation, a cause of thrombosis, atherosclerosis, and myocardial infarction.

Acknowledgments

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