



## Comparison of Antiplatelet Activities of Green Tea Catechins

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**ABSTRACT** – We have previously reported that green tea catechins (GTC) displayed potent antithrombotic effect, which was due to the antiplatelet activity. In the present study, the antiplatelet activity of each green tea catechin components was compared *in vitro*. Galloylated catechins including (-)-epigallocatechin gallate (EGCG), (-)-gallocatechin gallate (GCG), (-)-epicatechin gallate (ECG) and (-)-catechin gallate (CG), significantly inhibited collagen (5 µg/mL)-induced rabbit platelet aggregation with IC<sub>50</sub> values of 79.8, 63.0, 168.2 and 67.3 µM, respectively. EGCG, GCG and CG also significantly inhibited arachidonic acid (AA, 100 µM)-induced rabbit platelet aggregation with IC<sub>50</sub> values of 98.9, 200.0 and 174.3 µM, respectively. However catechins without gallate moiety showed little inhibitory effects against rabbit platelet aggregation induced by collagen or AA compared with galloylated catechins. These observations suggest that the presence of gallate moiety at C-3 position may be essential to the antiplatelet activity of catechins and the presence of B ring galloyl structure may also contribute to the antiplatelet activity of GTC. In line with the inhibition of collagen-induced platelet aggregation, EGCG caused concentration-dependent decreases of cytosolic calcium mobilization, AA liberation and serotonin secretion. In contrast, epigallocatechin (EGC), a structural analogue of EGCG lacking a galloyl group in the 3' position, although slightly inhibited collagen-stimulated cytosolic calcium mobilization, failed to affect other signal transductions as EGCG in activated platelets. Taken together, these observations suggest that the antiplatelet activity of EGCG may be due to inhibition of arachidonic acid liberation and inhibition of Ca<sup>2+</sup> mobilization and that the antiplatelet of EGCG is enhanced by the presence of a gallate moiety esterified at carbon 3 on the C ring.

**Key words:** Catechin, epigallocatechin gallate (EGCG), antiplatelet activity, arachidonic acid liberation, calcium mobilization

Platelet activation plays an important role in the initiation and maintenance of atherosclerosis and the thrombotic complications of atheroma.<sup>1-5)</sup> When blood vessels become damaged, subendothelial macromolecules such as collagen and thrombin, which are exposed or generated at the site of damage, stimulate platelet activation and initiate of hemostasis. This results in platelet shape change and formation of thromboxane (TX) A<sub>2</sub> and the release or secretion of proactivatory substances that activate and recruit platelets to the developing thrombus. Thus, inhibition of platelet aggregation may be a promising approach for the prevention of thrombosis.

Platelet adhesion to collagen leads to phosphatidic acid

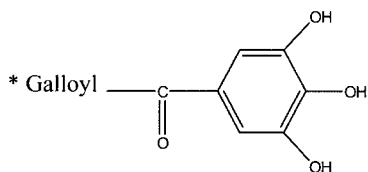
formation and calcium mobilization,<sup>6)</sup> which indicates that phospholipase (PL) C is activated in adherent platelets. Indeed, phosphoinositide metabolism and associated Ca<sup>2+</sup> mobilization in response to platelet adhesion to collagen appears to be associated with tyrosine phosphorylation of PLCγ2.<sup>7,8)</sup> Increases in [Ca<sup>2+</sup>]<sub>i</sub> can activate a Ca<sup>2+</sup>-sensitive cytosolic PLA<sub>2</sub> in platelets,<sup>9)</sup> leading to liberation of arachidonic acid (AA) from membrane phospholipids.<sup>10-11)</sup> Then the liberated AA is subsequently catalyzed by cyclooxygenase (COX) and thromboxane synthase to TXA<sub>2</sub>, which is a potent platelet aggregator and vasoconstrictor.<sup>12-14)</sup> Accordingly, agents with inhibition of the Ca<sup>2+</sup> mobilization and TXA<sub>2</sub> formation in platelets may suppress the platelet aggregation.

Epidemiological studies indicate that intake of green tea is associated with a lower risk of cardiovascular disease.<sup>15-18)</sup> Green tea constituents, GTC was consisted of eight catechins; (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epicatechin (EC), (-)-gallocatechin gallate (GCG), (-)-catechin gallate (CG), (-)-

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**Table 1.** Chemical structures of green tea catechin components

GTC components	Position		
	R <sub>1</sub>	R <sub>2</sub>	
(-) Epigallocatechin gallate	OH	Galloyl	
(-) Epigallocatechin	OH	OH	
(-) Epicatechin gallate	H	Galloyl	
(-) Epicatechin	H	OH	
(-) Gallocatechin gallate	OH	Galloyl	
(-) Gallocatechin	OH	OH	
(-) Catechin gallate	H	Galloyl	
(+) Catechin	H	OH	



gallocatechin (GC) and (+)-catechin (C) (Table 1). Several studies have demonstrated that gallate moiety is important in biological activities such as antioxidant, antibacterial, anticarcinogenic and blood cell protective effects. Epigallocatechin gallate (EGCG), a major component of the green tea catechins (GTC), is the most extensively studied tea polyphenol.<sup>19, 20</sup> It has been reported that EGCG has many biological functions, including antioxidant,<sup>21</sup> antimutagenic<sup>22</sup> and anticarcinogenic effects.<sup>23, 24</sup> In our previous study, we have reported that GTC and EGCG display potent antithrombotic effect *in vivo*, and this may be due to its antiplatelet rather than anticoagulant effect.<sup>25, 26</sup> More recently, it was reported that antiplatelet effect of GTC may be due to the inhibition of TXA<sub>2</sub> formation through the suppressions of AA liberation and TXA<sub>2</sub> synthase activity.<sup>27</sup>

In this study, we compared the antiplatelet activity of these eight GTC components on rabbit platelet to investigate whether gallate moiety is implicated in the different antiplatelet activity of galloylated catechins. In order to further clarify the antiplatelet activity of EGCG, we undertook to determine the effects of EGCG as compared to EGC on platelet

aggregation, arachidonic acid cascade and cytosolic calcium mobilization.

## Materials and methods

### Materials

(-)-Epigallocatechin gallate, (-)-epigallocatechin, (-)-gallocatechin gallate, (-)-gallocatechin, (-)-epicatechin gallate, (-)-catechin gallate, (-)-epicatechin and (+)-catechin purified from green tea were obtained from Sigma Chemical Co. (St Louis, MO, USA); the purities of these catechins used in the present study were all higher than 98%. Collagen and arachidonic acid were purchased from Chrono-Log Co. (Havertown, PA, USA). Anti-PLC $\gamma$ 2 polyclonal antibody was purchased from Santa Cruz Biotechnology (Autogen Bioclear UK Ltd, Calne, Wilts, UK), and U73122 was purchased from Tocris (Avonmouth Bristol, BS, UK). TXB<sub>2</sub> enzyme immunoassay (EIA) kit and antirabbit-horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Amersham Pharmacia (Little Chalfont, Buckinghamshire, UK). U46619 (9,11-dideoxy-9 $\alpha$ ,11 $\alpha$ -methano-

epoxy-prostaglandin  $F_{2\alpha}$ ),  $TXB_2$ ,  $PGD_2$  and  $PGH_2$  were from Cayman Chemical Co. (Ann Arbor, MI, USA). Indomethacin, imidazole, imipramine and fura-2/AM were from Sigma Chemical Co. (St Louis, MO, USA). [ $^3H$ ]arachidonic acid (250  $\mu$ Ci/mmol) was from New England Nuclear (Boston, MA, USA). Other reagents were of analytical grade.

### Animals

Male New Zealand white rabbits were purchased from the Samtako Bio Korea Inc. (Osan, Gyunggi, Korea), and acclimated for at least one week at a temperature of  $24 \pm 1$  °C and a humidity of  $55 \pm 5\%$  with free access to a commercial pellet diet obtained from the Samyang Co. (Wonju, Kangwon, Korea) and drinking water before experiments. Animal experiments were carried out in accordance with "Guide for the Care and Use of Laboratory Animals (Chungbuk National University, Korea)".

### Preparation of washed rabbit platelets

The preparation of washed rabbit platelets was performed as previously described.<sup>27</sup> In brief, rabbit blood was withdrawn from the ear artery vessel and collected directly into 0.15 (v/v) of anticoagulant citrate dextrose solution containing 0.8% citric acid, 2.2% trisodium citrate and 2% dextrose (w/v). Platelet rich plasma (PRP) was prepared by centrifugation at  $230 \times g$  for 10 min at room temperature. Platelets were sedimented by centrifugation of PRP at  $2,100 \times g$  for 10 min, and then washed twice with HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM  $MgCl_2$ , 5.6 mM glucose, 0.35% bovine serum albumin and 3.8 mM HEPES, pH 6.5) containing 0.4 mM EGTA. After centrifugation, the pellets were resuspended in HEPES buffer (pH 7.4). The platelet concentration was counted using a Coulter Counter (Coulter Electronics, Hialeah, FL, USA) and adjusted to  $4 \times 10^8$  platelets/mL.

### Measurement of platelet aggregation *in vitro*

Platelet aggregation was measured as previously described.<sup>28</sup> Briefly, washed platelet suspensions were incubated at 37°C in the aggregometer with stirring at 1,000 rpm, and then various concentrations of EGCG, GCG, ECG and CG were added. Platelet aggregation was induced by the addition of collagen (5  $\mu$ g/mL) and arachidonic acid (100  $\mu$ M), respectively. The resulting aggregation measured as the change in light transmission was recorded for 10 min.

### Measurement of serotonin secretion

Serotonin concentration was determined by the fluorimetric method of Holmsen and Dangelmaier.<sup>29</sup> To prevent the reuptake of secreted serotonin from the dense-granule contents, imipramine (5  $\mu$ M) was pretreated in the washed platelet

suspension. Washed platelets were pretreated with EGCG or EGC at 37°C for 3 min, and then collagen (5  $\mu$ g/mL) was added. After 5 min, the reaction was stopped by the addition of 5 mM EDTA in ice and then the supernatant was centrifuged at  $12,000 \times g$  for 2 min. The supernatant was mixed with 6 M trichloroacetic acid (TCA) and centrifuged at  $12,000 \times g$  for 2 min. A 0.6 mL aliquot of TCA supernatant was mixed with 2.4 mL of the solution (0.5% o-phthalaldehyde in ethanol diluted 1:10 with 8 N HCl), placed in a boiling water bath for 10 min, and then cooled in ice. The excess TCA was extracted with chloroform and fluorescence was measured at the wavelength of excitation (360 nm) and emission (475 nm). Serotonin creatinine sulfate was used as standard solution to calculate the extent of serotonin release.

### Measurement of arachidonic acid liberation

The AA liberation assay was performed as previously described.<sup>30</sup> In brief, PRP was incubated with [ $^3H$ ]AA (1  $\mu$ Ci/mL) at 37°C for 1.5 h, and then washed as described above. The [ $^3H$ ]AA labeled platelets ( $4 \times 10^8$  platelets/mL) were pretreated with 100  $\mu$ M BW755C (3-amino-1-[m-(trifluoromethyl)-phenyl]-2-pyrazoline, a cyclooxygenase (COX) and lipoxygenase (LOX) inhibitor) and various concentration of EGCG or EGC at 37°C for 3 min in the presence of 1 mM  $CaCl_2$ , and then stimulated by the addition of collagen (50  $\mu$ g/mL). The reaction was terminated by addition of chloroform:methanol:HCl (200:200:1, v/v/v). After the mixture was vortexed, 5 mM EGTA (containing 0.1 M KCl) was added. Then, samples were centrifuged at  $2,100 \times g$  for 10 min and separated upper phase was removed and evaporated to dryness under nitrogen. Residues were dissolved in chloroform:methanol (2:1, v/v) and were applied to silica gel G thin layer chromatography (TLC) plates (Analtech, Delaware, USA). The plates were developed in petroleum ether:diethyl ether:acetic acid (40:40:1, v/v/v). The area corresponding to arachidonic acid or diacylglycerol was scraped off and the radioactivity was determined by liquid scintillation counting.

### Measurement of $[Ca^{2+}]_i$

The measurement of  $[Ca^{2+}]_i$  was performed as previously described.<sup>31</sup> Briefly, fura 2/AM (final concentration: 3  $\mu$ M) was added to platelet-rich plasma, and the mixture was incubated for 30 min at 37°C. After washing, fura 2/AM-loaded platelets were resuspended in HEPES buffer containing 1 mM  $CaCl_2$  and adjusted at a concentration of  $4 \times 10^8$  platelets/mL. Platelets were incubated with 200  $\mu$ M EGCG or EGC for 3 min, then collagen (5  $\mu$ g/mL) or thapsigargin (1  $\mu$ M) was added. The measurements of  $[Ca^{2+}]_i$  were performed at room temperature in an MSIII fluorometer (Photon Technology International, Princeton, NT, USA)

using excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm.  $[Ca^{2+}]_i$  values were calculated by using the general formula described by Grynkiewicz.<sup>32)</sup>

### Statistical analysis

The experimental results were expressed as mean  $\pm$  S.D. A one-way analysis of variance (ANOVA) was used for multiple comparisons followed by Dunnett, and the data were considered significant with a probability less than 0.05.

## Results

### Effect of GTC on rabbit platelet aggregation *in vitro*

Galloylated catechins (EGCG, ECG, GCG, CG) concentration-dependently inhibited the rabbit platelet aggregation stimulated by collagen (5  $\mu$ g/mL) or arachidonic acid (AA, 100  $\mu$ M) *in vitro*, whereas non-galloylated catechins (EGC, GC, EC, C) showed little inhibitory effects compared with the galloylated catechins. As shown in Table 2 and Fig. 1, GCG showed the most potent inhibitory effect against the collagen-induced rabbit platelet aggregation, followed by EGCG, GCG, ECG and CG with  $IC_{50}$  values of 79.8, 63.0, 168.2 and 67.3  $\mu$ M, respectively. On the other hand, EGCG showed the most potent inhibition against the AA-induced platelet aggregation, followed by EGCG, GCG, and CG with  $IC_{50}$  values of 98.9, 200.0 and 174.3  $\mu$ M, respectively. Among the galloylated catechins, ECG showed the most weak inhibitory effect against both collagen- and AA-induced platelet aggregation. Treatment with non-galloylated catechins showed little inhibitory effects against collagen- or AA-induced rabbit platelet aggregation up to the concentration of 200  $\mu$ M. Aspirin, a selective COX inhibitor, significantly

**Table 2.** Effects of green tea catechin components on rabbit platelet aggregation

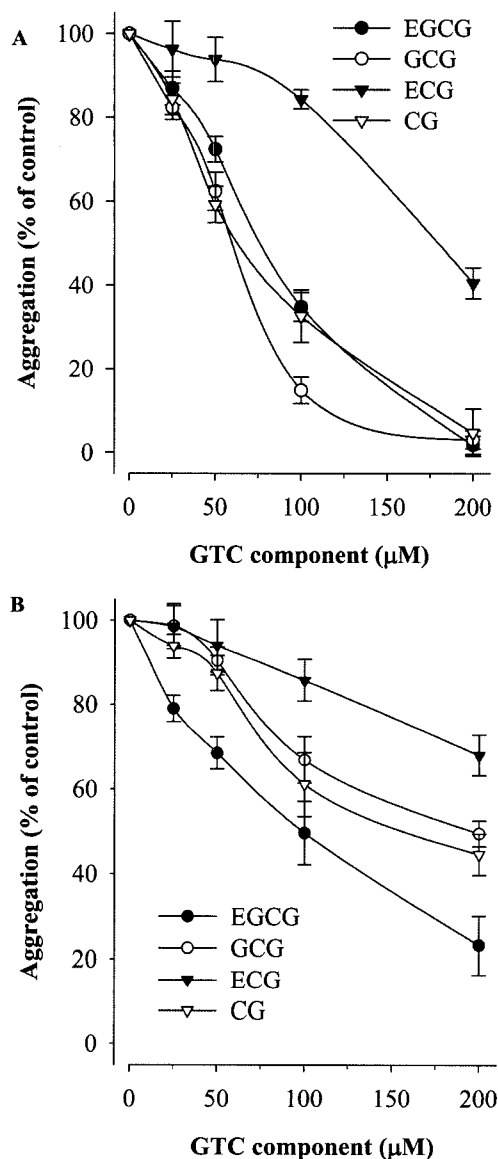
Catechins	Collagen (5 $\mu$ g/mL)		Arachidonic acid (100 $\mu$ M)	
	Inhibition % (100 $\mu$ M)	$IC_{50}$ ( $\mu$ M)	Inhibition % (100 $\mu$ M)	$IC_{50}$ ( $\mu$ M)
EGCG	65.2 $\pm$ 3.5	79.8	50.4 $\pm$ 7.5	98.9
GCG	84.5 $\pm$ 3.2	63.0	33.1 $\pm$ 5.5	200.0
ECG	15.7 $\pm$ 2.3	168.2	14.3 $\pm$ 4.9	>200
CG	67.4 $\pm$ 6.3	67.3	38.9 $\pm$ 7.6	174.3
EGC	43.5 $\pm$ 5.4	>200	3.0 $\pm$ 1.2	>200
GC	5.6 $\pm$ 6.1	>200	17.5 $\pm$ 6.5	>200
EC	7.2 $\pm$ 4.3	>200	7.6 $\pm$ 3.8	>200
C	1.2 $\pm$ 2.4	>200	2.1 $\pm$ 3.5	>200
Aspirin	66.2 $\pm$ 4.2	42.4	100	16.9

Washed rabbit platelets were incubated at 37°C in an aggregometer with stirring at 1,000 rpm, and then GTC component or aspirin was added. After 3 min preincubation, platelet aggregation was induced by addition of collagen (5  $\mu$ g/mL) and AA (100  $\mu$ M), respectively. Data are expressed as mean  $\pm$  SD (n=4).

inhibited the AA-induced rabbit platelet aggregation with an  $IC_{50}$  value of 16.9  $\mu$ M. Aspirin as a positive control also exhibited an inhibitory effect on collagen-induced platelet aggregation with an  $IC_{50}$  value of 42.4  $\mu$ M.

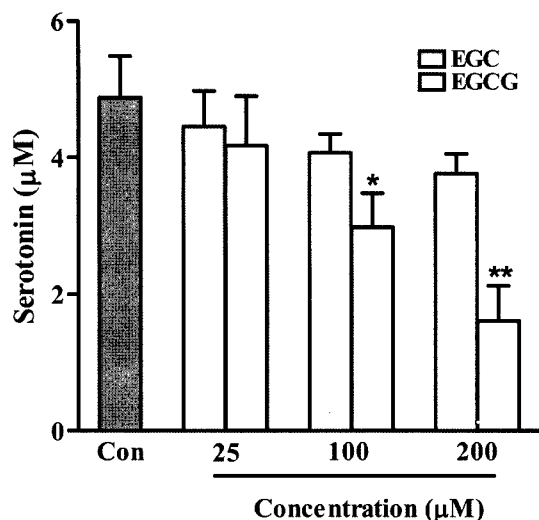
### Effect of GTC on serotonin release

The serotonin level in resting platelets was less than 0.2  $\mu$ M/ $4 \times 10^8$  cells and stimulated by 5  $\mu$ M/ $4 \times 10^8$  cells with addition of collagen (5  $\mu$ g/mL). As shown in Fig. 2, the

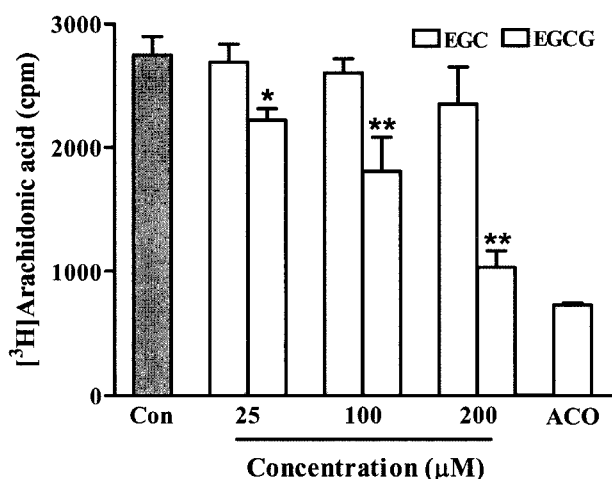


**Fig. 1.** Effects of green tea catechin components on rabbit platelet aggregation *in vitro*. Washed rabbit platelets were incubated at 37°C in an aggregometer with stirring at 1,000 rpm, and then GTC components were added. After 3 min preincubation, the platelet aggregation was induced by addition of collagen (5  $\mu$ g/mL, panel A) and arachidonic acid (AA, 100  $\mu$ M, panel B), respectively. The aggregation percentages present % of the maximal aggregation. Data are expressed as mean  $\pm$  S.D. (n=4).

pretreatment of EGCG significantly inhibited the release of serotonin by 14.4, 38.9 and 67.1% at concentration of 25, 100 and 200  $\mu\text{M}$ , while EGC only slightly inhibited at a concentration of 200  $\mu\text{M}$  (22.7%). This observation was



**Fig. 2.** Effects of EGCG and EGC on serotonin secretion in rabbit platelets. Washed rabbit platelet suspension was incubated with indicated concentration of EGCG or EGC for 3 min at 37°C. And then collagen (5  $\mu\text{g}/\text{mL}$ ) was added. After 5 min, the reaction was terminated by addition of 5 mM ice-cold EDTA and then the supernatant was obtained by centrifugation at 12,000 $\times$ g for 2 min. The serotonin concentration was determined by a fluorimetric method (Holmsen and Dangelmaier, 1989). Data were expressed as mean  $\pm$  S.D. (n=4). Significantly different from control at \*P<0.05, \*\*P<0.01

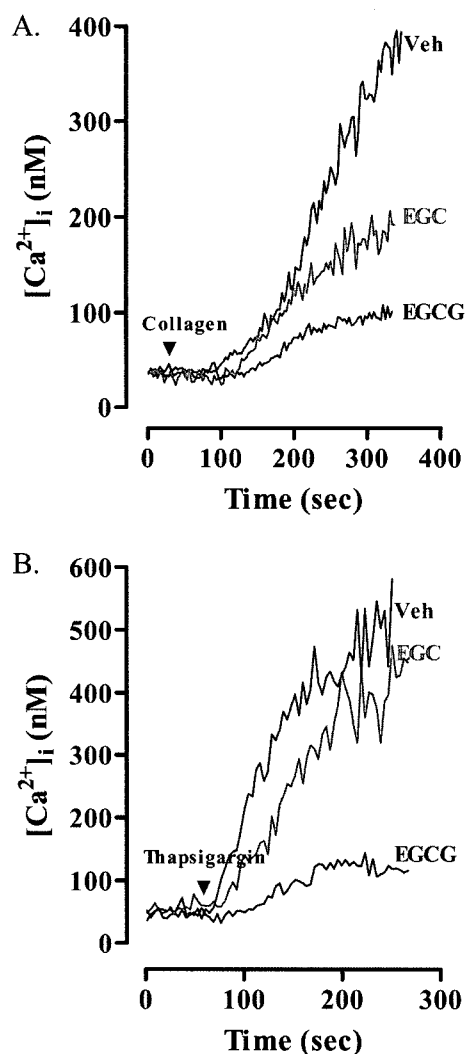


**Fig. 3.** Effects of EGCG and EGC on arachidonic acid liberation in rabbit platelets. [ $^3\text{H}$ ]arachidonic acid-labeled platelets were incubated with various concentrations of EGCG, EGC or AACOCF<sub>3</sub> (cPLA<sub>2</sub> inhibitor) at 37°C for 2 min in the presence of 50  $\mu\text{M}$  BW755C, and then stimulated with 50  $\mu\text{g}/\text{mL}$  collagen for 2 min. [ $^3\text{H}$ ]arachidonic acid liberated was determined as described in Materials and methods. Each value represents mean  $\pm$  S.D (n=3). Significantly different from control at \*P<0.05, \*\*P<0.01.

accompanied with the inhibition of platelet aggregation.

#### Effect of GTC on arachidonic acid liberation

Effects of EGCG and EGC on AA liberation were estimated by using [ $^3\text{H}$ ]AA-labelled platelets (Fig. 3). As shown in Fig. 3, AACOCF<sub>3</sub> (cPLA<sub>2</sub> inhibitor, 50  $\mu\text{M}$ ), as a positive control significantly inhibited AA liberation (28.3% of control), whereas thioetheramide-PC (sPLA<sub>2</sub> inhibitor, 50  $\mu\text{M}$ ) did not have any effects. This result, indeed, showed that collagen-induced AA liberation in the present assay condition was mediated mainly by cPLA<sub>2</sub>. Pretreatment of EGCG significantly decreased AA release (67.5% of control), while EGC inhibited weakly than EGCG at a concentration of 200  $\mu\text{M}$  (14.5% of control).



**Fig. 4.** Effects of EGCG and EGC on [ $\text{Ca}^{2+}$ ]<sub>i</sub> mobilization in rabbit platelets caused by collagen. The platelets suspension contained 1 mM CaCl<sub>2</sub>. Fura 2/AM-loaded platelets were pre-incubated with DMSO (0.5%, Veh), EGCG or EGC (200  $\mu\text{M}$ ) for 3 min and then collagen (5  $\mu\text{g}/\text{mL}$ ) or thapsigargin (1  $\mu\text{M}$ ) was added. The traces are representative of three similar experiments.

### Effect of GTC on calcium influx

In our previous study, green tea catechins (GTC) inhibited calcium increase and platelet aggregation in platelet-rich plasma.<sup>26)</sup> Whether EGCG and EGC can inhibit the  $[Ca^{2+}]_i$  increase was also observed in fura 2/AM-loaded rabbit platelets. EGCG (200  $\mu$ M) actually suppressed the  $[Ca^{2+}]_i$  elevation by collagen (5  $\mu$ g/mL), whereas EGC (200  $\mu$ M) had weakly inhibitory effect (Fig. 4A). As shown in Fig. 4B, EGCG also reduced  $Ca^{2+}$  mobilization induced by thapsigargin (1  $\mu$ M), which induces a  $Ca^{2+}$  efflux from the endoplasmic reticulum by inhibiting the endoplasmic reticulum ATPase, triggering a cellular massive influx of the ion.<sup>34,35)</sup> On the contrary, EGC was not effective to inhibit the collagen- or thapsigargin-induced  $[Ca^{2+}]_i$  increase in comparison to EGCG.

## Discussions

We previously reported that GTC displayed the antithrombotic effect through the antiplatelet aggregation.<sup>25)</sup> It was also reported that the antiplatelet activity of GTC could be mediated by inhibition of cytoplasmic  $Ca^{2+}$  increase.<sup>26)</sup> In the present study, Galloylated catechins (EGCG, ECG, GCG, CG) concentration-dependently inhibited the rabbit platelet aggregation stimulated by collagen (5  $\mu$ g/mL) or arachidonic acid (AA, 100  $\mu$ M) *in vitro*, whereas non-galloylated catechins (EGC, GC, EC, C) showed little inhibitory effects compared with the galloylated catechins. As shown in Table 2 and Fig. 1, GCG showed the most potent inhibitory effect against the collagen-induced rabbit platelet aggregation, followed by EGCG, GCG, ECG and CG with  $IC_{50}$  values of 79.8, 63.0, 168.2 and 67.3  $\mu$ M, respectively. On the other hand, EGCG showed the most potent inhibition against the AA-induced platelet aggregation, followed by EGCG, GCG, and CG with  $IC_{50}$  values of 98.9, 200.0 and 174.3  $\mu$ M, respectively. Among the galloylated catechins, ECG showed the most weak inhibitory effect against both collagen- and AA-induced platelet aggregation. Treatment with non-galloylated catechins showed little inhibitory effects against collagen- or AA-induced rabbit platelet aggregation up to the concentration of 200  $\mu$ M. Aspirin, a selective COX inhibitor, significantly inhibited the AA-induced rabbit platelet aggregation with an  $IC_{50}$  value of 16.9  $\mu$ M. Aspirin as a positive control also exhibited an inhibitory effect on collagen-induced platelet aggregation with an  $IC_{50}$  value of 42.4  $\mu$ M. In our *in vitro* antiplatelet assay by using of human platelets, galloylated catechins also showed significant inhibitions against both collagen- and AA-induced platelet aggregations in order of GCG > CG > EGCG > ECG, whereas non-galloylated catechins showed little inhibitory effects compared with the galloylated catechins (data not shown).

In many *in vitro* studies including our present study, the concentration of catechins seems to be too high to be physiologically relevant for tea drinkers, since plasma concentration of EGCG or other catechins reaches only 0.1-0.3  $\mu$ M after moderate green tea consumption.<sup>16)</sup> The relation between *in vitro* and *in vivo* beneficial effects of green tea is not clear. However, many epidemiological studies indicate that moderate consumption of green tea might prevent the incidence of cardiovascular disease and proliferative diseases such as cancer and atherosclerosis in human.<sup>6-9,17,18)</sup> In addition, GTC and EGCG actually prevented platelet aggregation *ex vivo* and death due to pulmonary thrombosis and prolonged the tail bleeding time in mice *in vivo*.<sup>10)</sup>

Our findings are in agreement with the notion that galloylated catechins show more effective antioxidant, antibacterial, anticarcinogenic and blood cell protective activities than their homologues lacking gallate moiety.<sup>12-15)</sup> These results suggest that the presence of gallate moiety at C-3 position may be essential to the antiplatelet activity and the presence of B ring galloyl structure may also contribute to the antiplatelet activity of GTC.

We provided evidence that antiplatelet activity and mechanism of polyphenolic compounds EGCG may be due to the inhibition of arachidonic acid liberation and cytosolic calcium mobilization, and antiplatelet activity of EGCG is enhanced by the presence of a gallate moiety esterified at carbon 3 on the C ring compared to EGC.

Many of the biological effects of green tea catechins have also been related to the presence of gallate moiety in their structure. As shown in Fig. 1, EGCG potently inhibited the collagen- and AA-induced aggregation in a concentration-dependent manner. In contrast, EGC only weakly inhibited the collagen-induced aggregation, while showed little effect on platelet aggregation induced by other two inducers. These results were also agreed with other reports that EGCG and ECG appear more effective antioxidant, antibacterial, anticarcinogenic activities than EGC.<sup>36-39)</sup> Generally, we have observed that gallated catechins including EGCG, GCG, ECG and CG present more potent inhibitory effects on rabbit platelet aggregation than catechins without gallate moiety on C ring. EGCG and EGC also present collagen-induced serotonin release at similar concentrations as platelet aggregation (Fig. 2).

$TXA_2$  is an important mediator of the release reaction and aggregation of platelets.<sup>14)</sup> When platelets are activated by collagen or thrombin,  $TXA_2$ , a potent platelet-aggregating agent, is formed from AA liberated from membrane phospholipids by  $Ca^{2+}$ -dependent phospholipase  $A_2$  or diglyceride lipase<sup>40,41)</sup> in stimulated platelet. Since the intracellular concentration of free AA is low, the release of AA is thought to be the rate-limiting step in the formation of prostaglandin

(PG) and the other eicosanoids including TXA<sub>2</sub>. As shown in Fig. 2, the AA liberation was significantly inhibited by EGCG in a dose-dependent manner, whereas EGC had little effect. Collagen-induced AA liberation is thought to be mediated by the cytosolic phospholipase A<sub>2</sub> rather than the secretory phospholipase A<sub>2</sub>. It is supported by the findings that stimulus-induced arachidonic acid liberation increases with increasing expression of the cytosolic enzyme but not the secretory enzyme,<sup>42)</sup> and that the addition of secretory phospholipase A<sub>2</sub> to intact or stimulated rabbit platelets does not cause any response.<sup>43)</sup> DAG is formed upon breakdown of membrane phospholipids by phospholipase C.<sup>44)</sup> Accumulation of DAG stimulates protein kinase C (PKC), which is involved in various aspects of platelet activation.<sup>45)</sup> EGCG also significantly inhibit the collagen- and thapsigargin, a Ca<sup>2+</sup>-ATPase inhibitor-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> in platelet. Considering that AA by cPLA<sub>2</sub> is [Ca<sup>2+</sup>]<sub>i</sub>-dependent process, it is suggested that [Ca<sup>2+</sup>]<sub>i</sub>-lowering effects of EGCG may be associated with its inhibitory effect on AA liberation, although the direct effect on cPLA<sub>2</sub> was not investigated in this study.

Taken together, these observations suggest that the antiplatelet activity of EGCG may be due to inhibition of arachidonic acid liberation and inhibition of Ca<sup>2+</sup> mobilization and that the antiplatelet of EGCG is enhanced by the presence of a gallate moiety esterified at carbon 3 on the C ring.

## 요 약

저자들은 녹차 카테킨(GTC)이 강한 항 혈전 작용을 나타내며, 이는 항 혈소판 활성화에 의한 것임을 보고한 바 있다. 본 연구에서는, 8가지 녹차 카테킨 성분들의 항 혈소판 활성을 비교하였다. 실험결과, 갈레이트(gallate) 구조를 갖는 카테킨들(EGCG, GCG, ECG, CG)은 콜라겐(5 µg/ml)으로 유도한 토끼 혈소판 응집능을 강하게 억제하였으며, 50% 억제농도(IC<sub>50</sub>)는 각각 79.8, 63.0, 168.2, 67.3 µM이었다. 또한 EGCG, GCG, CG는 아라키돈산(AA, 100 µM)으로 유도한 토끼 혈소판 응집능을 억제하였고, 50% 억제농도(IC<sub>50</sub>)는 각각 98.9, 200.0, 174.3 µM이었다. 반면에, 갈레이트 구조를 가지지 않는 카테킨들은 혈소판 응집능 억제 효과가 매우 약했다. 이 결과는 항 혈소판 활성화에서 카테킨들의 C-3 위치의 갈레이트 구조의 존재가 매우 중요하다는 것과 카테킨들과 B-ring 갈레이트 구조의 존재 또한 녹차카테킨의 항 혈소판 활성화에 중요한 작용한다는 것을 의미한다. 그리고, EGCG는 농도 의존적으로 세포내 칼슘 생성과 아라키돈산의 생성을 억제시켰는데, 이는 혈소판 응집능의 억제와 일치하였다. 반면에, EGC는 세포내 칼슘 및 다른 혈소판 활성화 기전에 아무런 영향이 없었

다. 이들 결과는 EGCG의 항 혈소판 활성화는 C-ring에서 carbon 3 자리의 에스테르화 된 갈레이트 구조의 존재에 의해서 강화된 항 혈소판 작용으로, 아라키돈산 생성과 세포내 칼슘 생성을 억제하는 효과에 기인한 것이라 사료된다.

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