Effects of Green Tea Catechin on Phospholipase A₂ Activity and Antithrombus in Streptozocin Diabetic Rats

Jeong-Ah YANG, Jeong-Hwa CHOI and Soon-Jae RHEE*

Department of Food Science and Nutrition, Catholic University of Taegu-Hyosung, Kyungsan-si, Kyungbuk, 713-702, Korea
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Summary The purpose of this study was to investigate the effects of dietary green tea catechin on phospholipase A₂ (PLA₂) activity and the antithrombotic reaction of platelets in streptozocin (STZ)-diabetic rats. Sprague-Dawley male rats weighing 100 ± 10 g were randomly divided into one normal and three STZ-diabetic groups, which were subdivided into catechin-free group (DM-0C), 0.5% catechin group (DM-0.5C) and 1% catechin group (DM-1C). The activity level of platelet phospholipase A₂ was higher in the diabetic groups than in the normal group, while it was lower in DM-0.5C and DM-1C than in DM-0C. The activity of platelet cyclooxygenase in DM-0C was 1.1-fold as high as in the normal group, but was significantly reduced by catechin supplementation. The platelet thromboxane A₂ (TXA₂) formation became higher in DM-0C as compared to the normal group, but not in DM-0.5C and DM-1C. The synthesis of aortic prostacyclin (PGI₂) was lower in DM-0C and DM-0.5C than in the normal group. The PGI₂/TXA₂ ratio was decreased to 55% in DM-0C, but was restored by catechin supplementation. These results indicate that STZ-diabetic rats are sensitive to platelet aggregation and thrombosis, and that the abnormality can be improved by dietary catechin.

Key Words tea catechin, phospholipase A₂, antithrombus, diabetic rats

In severe diabetic state, the abnormality in electrolyte or energy metabolism is accompanied by various complications such as neuropathic lesion, ophthalmopathic disease and vascular disease (1).

It has been known that the pathogenesis of vascular disease such as coronary cardiopathic disease and arteriosclerosis is two to six times higher in diabetics than that in normal subjects (2).

Increased platelet insertion in the blood vessel wall and platelet aggregation are reported to be attributable to these vascular disorders in diabetic state (3–6).

Furthermore, it has been reported that enhanced platelet agglutinability results in an increase in the production of thromboxane A₂ (TXA₂), through the deacylated

*To whom correspondence should be addressed.
membrane phospholipid and activated arachidonic acid (AA) metabolism, while
decreasing the formation of antiaggregative agents, such as prostacyclin (PGI₂),
functioning as a counteractive to TXA₂ (7, 8).

Many reports have pointed out that an imbalance between PGI₂ and TXA₂
has a significant effect on platelet aggregation in the diabetic state (7). This is
because PGI₂ synthetase is inhibited by lower concentrations of lipid peroxides
than TX synthetase.

Accordingly, it is important for their ratio to be restored to the normal level
to prevent diabetic complications (9).

Phospholipase A₂, cyclooxygenase and TX synthetase are enzymes essential
to form TXA₂. The inhibition of TXA₂ production and the elevation of PGI₂
production are effective in controlling platelet agglutinability.

Since such a cyclooxygenase blocker as aspirin suppresses the formation of
TXA₂ and PGI₂, its preventive effect on thrombus is practically unexpectable (10).
For this reason, it will be necessary to search substances that can specifically inhibit
TX synthetase or compete with platelet membrane TXA₂ receptor or elevate the
production of PGI₂.

Meanwhile, it has been known that green tea catechin, among natural
substances, has several pharmacological functions, such as hypocholesterolemic
action (11), anti-oxidation (12), and inhibition of platelet agglutinability (13).

A previous report has referred to in vitro inhibition of thrombus or platelet
aggregation by green tea catechin (13). However, there is little information on its
in vivo effect, and at present, no reports are available about the action of catechin
as an inhibitor of platelet agglutinability.

In this study, green tea catechin was examined for its effects on the activation
of phospholipase A₂ from epicyte phospholipid and imbalance of PGI₂/TXA₂ in
order to investigate the most critical step involved in platelet aggregation in a
diabetic state. Also, the potentiality of catechin inhibition to platelet agglutinability
in the diabetic state was investigated in connection with phospholipase A₂,
cyclooxygenase and TX synthetase in the AA cascade.

MATERIALS AND METHODS

Experimental animals and diets. After male Sprague-Dawley rats weighing
about 70 g were acclimatized to our facility for 1 wk, they were allotted to a
normal group and three experimental diabetic groups. The four groups (n = 10)
were maintained on their respective experimental diets for 4 wk, of which the
diabetic groups were classified as DM-0C (catechin-free), DM-0.5C (catechin, 0.5%)
and DM-1C (catechin, 1%), respectively, according to the level of catechin
supplementation. Crude catechin powder was prepared by the method of Matsuzaki
and Hata (12), and used as such. Catechin content in the crude powder and dietary
composition of the basal diet are shown in Tables 1 and 2. This experimental design
was approved by the Committee of Catholic University of Taegu-Hyosung for care
Table 1. Catechin contents in crude catechin preparation from green tea.

| Catechin in dry powder | (%) on a dry weight basis |
|------------------------|--------------------------|
| Epigallocatechin        | 24.2                     |
| Epicatechin            | 7.0                      |
| Epigallocatechin gallate | 45.3                   |
| Epicatechin gallate    | 10.9                     |
| **Total**              | **87.5**                 |

Catechin was determined by HPLC in a manner previously described (12).

Table 2. Composition of basal diet.

| Ingredient                        | Composition (g/kg diet) |
|-----------------------------------|-------------------------|
| α-Corn starch^1                   | 668                     |
| Casein^2                          | 180                     |
| dl-Methion^e^3                    | 2                       |
| Corn oil^4                        | 50                      |
| Vitamin mix^5                     | 10                      |
| Mineral mix^6                     | 40                      |
| Cellulose^7                       | 50                      |
| kcal/kg                            | 3.850                   |

1 Pung Jin Chem. Co., Seoul, Korea.
2 Lactic casein, 30 mesh, Newzealand Daily Board, Wington, N.Z.
3 Sigma Chem., St. Louis, Missouri, USA.
4 Dong Bang Oil Co., Seoul, Korea.
5 AIN-76 likeness (g/kg mixture): CaHPO₄·2H₂O 500, NaCl 74, K₃C₆H₅O₇·H₂O 220, K₂SO₄ 52, MgO 24, MgCO₃ (45-48% Mn) 3.5, Fe citrate (16-17% Fe) 6, Zn carbonate (70% Zn) 1.6, Cu carbonate (53-55% Cu) 0.3, KIO₃ 0.01, Na₂SeO₃·5H₂O 0.01, Cr₃(PO₄)₂·12H₂O 0.55; filled up to 1,000 with sucrose.
6 AIN-76 likeness (mg/kg mixture): thiamin·HCl 600, riboflavin 600, pyridoxine·HCl 700, nicotinic acid (nicotinamide in equivalent) 3,000, calcium pantothenate 1,600, folic acid 200, biotin 20, cyanocobalamine 1, retinyl palmitate or acetate 400,000 IU, dl-α-tocopheryl acetate 5,000 IU, cholecalciferol (100,000 IU) 2.5, menaquinone 5, filled up to 1,000 with sucrose.
7 Sigma Chem., St. Louis, Missouri, USA.

and use of laboratory animals.

Experimental diabetes. Diabetes was experimentally induced by intravenous injection of streptozotocin (STZ, 55 mg/kg BW) in citrate buffer (pH 4.3) into the tail vein. The rats whose blood-glucose level was over 16.7 mmol/L after 6 d were
Separation of platelet.

Preparation of platelet-rich plasma: Blood was collected from the abdominal aorta under anesthesia with diethyl ether and treated with a one-tenth volume of 3.8% (w/v) trisodium citrate solution as the anticoagulant, followed by centrifugation for 15 min at 200 \( \times \) g. The upper layer, deprived of erythrocyte and leukocyte, was once again centrifuged at 150 \( \times \) g for 15 min and the supernatant containing \( 1.5 \times 10^9 \) cells/L was used for the platelet aggregation test.

Preparation of washed platelet suspension: Platelet-rich plasma (PRP), obtained as above-mentioned, was washed with 26 mM Tris-HCl of pH 7.4 containing 130 mM NaCl, 2 mM EDTA, and resuspended in 0.03% bovin serum albumin (BSA)-containing Tyrodes buffer (150 mM NaCl, 0.55 mM NaH$_2$PO$_4$, 7 mM NaHCO$_3$, 2.7 mM KCl, 0.5 mM MgCl$_2$, 5.6 mM glucose, 5 mM HEPES). The washed platelet suspension (WPS) was used for measurement of aggregation and prostaglandin (PG) formation.

Experiment on activity of phospholipase A$_2$. Blood, stirred with acid citrate dextrose (ACD) was centrifuged at 400 \( \times \) g for 15 min. Platelets obtained from the supernatant by centrifugation at 1,400 \( \times \) g for 15 min were supplemented with 2.5 mL of platelet-poor plasma (PPP). Two hours after incubation at 37°C with 120 \( \mu \)L of \([^3H]AA\) (4.68 \( \times \) 10$^8$ Bq) according to the method of Moon et al (14), the platelet suspension was diluted with 15 mL of PPP and centrifuged at 1,400 \( \times \) g. The resulting \([^3H]AA\)-labeled platelets were blended in 5 mL of Tris-Tyroid buffer containing EDTA, centrifuged at 1,400 \( \times \) g for 15 min, and suspended with 1–2 mL of Tris-Tyroid buffer excluding EDTA. The platelet suspension (1 \( \times \) 10$^6$ cell/mL) was incubated in 50 \( \mu \)L of 200 \( \mu \)M propyl gallate and used for measurement of phospholipase A$_2$ activity. The sample was incubated at 37°C for 5 min, in 50 \( \mu \)L of thrombin (1 unit/mL), and then centrifuged at 400 \( \times \) g in 5 \( \mu \)L of 1.5 M formaldehyde. The pellet was mixed with 0.5 mL of distilled water, 0.6 mL of CHCl$_3$, and 1.2 mL of MeOH, followed by the addition of 1.2 mL of CHCl$_3$. After the lower layer was evaporated under a stream of nitrogen gas, 300 \( \mu \)L of CHCl$_3$ was added to the residue. Two-thirds of the resulting suspension (200 \( \mu \)L) was analyzed by thin-layer chromatography using ethylacetate/isoctane/acetic acid/water (90:50:20:10) as the developing solvent. Radioactivity of the arachidonic acid on the TLC plate was measured with a liquid scintillation counter.

Measurement of cyclooxygenase activity. Four-hundred-and-ninety microliters of the platelet suspension containing 1.5 \( \times \) 10$^9$ cells/mL was mixed with 10 \( \mu \)L of AA (final concentration 10 \( \mu \)M). After incubation at 37°C for 6 min, 50 \( \mu \)L of indomethacin (120 \( \mu \)M) containing 2 mM EDTA was added and 0.5 mL of the mixture was centrifuged at 8,000 \( \times \) g for 2 min. The amount of TXA$_2$ formation by cyclooxygenase was measured using a RIA assay kit of Amersham Co. (Amersham TRK 780).

Assay for thromboxane B$_2$ and 6-keto prostaglandin F$_{1a}$ instead of TXA$_2$ and PGI$_2$. TXA$_2$ easily changes into a semi-stable form, ‘TXB$_2$.’ Thus, TXB$_2$ was
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determined as an indicator of TXA2 by RIA specific for TXB2 using an assay kit of Amersham Co. (Amersham TRK 780). WPS (1.5 x 10^9 cells/L) was subjected to reaction with either collagen (final concentration, 50 μg/mL) or thrombin (final concentration, 0.5 U/mL). The reaction was stopped by adding indomethacin (final concentration, 20 μM). The supernatant after centrifugation was used for the TXB2 assay.

Since PGI2 has a half-life period too short for measurement, 6-keto prostaglandin F1α (a physiologically stable metabolite) was determined instead of PGI2. Slices of aorta (about 2 cm) were incubated in 2 mL of 0.05 M (pH 7.4) Tris buffer at 37°C for 30 min to stimulate the production of 6-keto prostaglandin F1α, and then 4.8 M formic acid was added to the medium. After the extraction of fat with n-hexane, the aorta slices were air-dried and weighed. The content of 6-keto prostaglandin F1α was measured by the use of a commercially available RIA kit (Amersham TRK 790). The radioactivity was measured with a Packard liquid scintillation counter. The amount of 6-keto prostaglandin F1α production was expressed as pg/mg of dry aorta.

Determination of serum lipid peroxide. Lipid peroxide (malondialdehyde) contents in the serum were fluorometrically measured by a modified TBA method of Yagi (15).

Statistical analysis. Data were analyzed by ANOVA, and when significance was found by ANOVA, the differences among groups were evaluated by Tukey’s test and considered significant at p<0.05.

RESULTS AND DISCUSSION

Effects of catechin on phospholipase A2 and cyclooxygenase activities

The results of measuring PLA2 activity, defined as the first rate-limiting enzyme in the AA cascade, are shown in Fig. 1. The PLA2 activity level in the DM-0C group was increased by 42% as compared to that in the normal group, whereas the PLA2 activity levels in the DM-0.5C and DM-1C groups were increased by 34 and 35%, respectively, indicating that catechin-dosed groups are significantly inferior to the DM-0C group in platelet PLA2 activity.

Cyclooxygenase activity is graphically illustrated in Fig. 2. The cyclooxygenase activity level was increased by 106% in the DM-0C group as compared to the normal group. In contrast, those in the DM-0C and DM-1C groups were increased by 48 and 54%, respectively. This indicates that catechin dosage lowers cyclooxygenase activity.

Effects of catechin on platelet TXB2 or aortic α-keto prostaglandin F1α production

While TXB2 showed a high increment of 169% in the DM-0C group as compared to the normal group, TXB2 in the DM-0.5C and DM-1C groups was not significantly different from that in the normal group (Table 3).

The production of α-keto prostaglandin F1α from aorta was reduced by 31 and
Fig. 1. Effects of green tea catechin on the phospholipase A2 activity in STZ-diabetic rats. Values are the means ± SE (n = 10). Those with different superscript letters are significantly different at p < 0.05 by Tukey’s test.

Fig. 2. Effects of green tea catechin on the cyclooxygenase activity in STZ-diabetic rats. Values are the means ± SE (n = 10). Those with different superscript letters are significantly different at p < 0.05 by Tukey’s test.

13% in the DM-0C and DM-0.5C groups, respectively, as compared to the normal group, whereas it was increased by 25% in the DM-1C group. The PGI2/TXA2 ratio was decreased by 55% in the DM-0C group relative to the normal group.

Conversely, the PGI2/TXA2 ratios in the DM-0.5C and DM-1C groups were increased by 16 and 53%, respectively, their increments being significantly different not only from each other but also from that of the normal group.
Effects of catechin on serum lipid TBA values

The concentration of TBA-reacting substance (TBARS), an index of peroxidation in serum lipid, was increased by 151% in the DM-0C group as compared to that in the normal group and increased by 70 and 25% in the DM-0.5C and DM-1C groups, respectively (Table 4). With respect to the DM groups, the TBA values in the DM-0.5C and DM-1C groups decreased by 32 and 50%, respectively, compared to that in the DM-0C group.

Although the TBA value of HDL increased 1.5-fold as much in the DM-0C group as compared to the normal group, it was reduced by 14% in both the DM-0.5C and DM-1C groups as compared to that in the DM-0C group. The TBA value of LDL tended to increase with decreased catechin-dose amounts among the DM groups, and became 5-fold higher in the DM-0C group than in the normal group.

The prime object of this study was to examine the effect of green tea catechin on PLA2 activity or thrombus in diabetic rats. In a diabetic state, cardiovascular

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disease (16) is caused by increasing lipid peroxidation (17), and its subsequent lesion accompanies adhesion or aggregation (18) of platelets. Activation of the AA cascade is the major mechanism of platelets to increase their own function in the diabetic state. Additionally, it can be suggested that platelet aggregation and pathogenesis or its subsequent cardiovascular complication are dependent on both production of the AA cascade system components (TXA₂, PGI₂, PGD₂, etc.) and the activation of enzymes (phospholipase A₂, cyclooxygenase, thromboxane synthetase, etc.).

Lipid peroxides generated by physical and chemical stresses activate PLA₂, and thereby elevate the release of AA from the epicyte membrane (19). In this experiment, the activity of PLA₂ proved to be increased by 42% in diabetic groups as compared to that in the normal group. The increment of AA release by PLA₂ in the platelets of diabetic rats was diminished by a catechin dose inhibitory to PLA₂ activity. This is in agreement with previous observations that an increase of PLA₂ activity is attributable to an increment of TXA₂ production in diabetics (20), and that hydroxybrazillin, a natural substance inhibitory to PLA₂, actually suppresses the formation of TXA₂ (14).

The production of TXA₂, is mediated by TX synthetase and cyclooxygenase is activated in the process of AA metabolism. Although aspirin is effective in blocking cyclooxygenase, this vehicle cannot be expected to restore the ratio of PGI₂/TXA₂ as effectively as an improved agent (10) because it concurrently inhibits the production of TXA₂ as well as PGI₂ (10).

The activity of cyclooxygenase is an accountable factor for the increment of platelet aggregability in the diabetic state, as it showed a significant increase in the DM-0C group relative to the normal group. This increased activity of cyclooxygenase was significantly diminished in the DM-0.5C group and was almost normalized in the DM-1C group.

TXA₂ has been regarded as the most potential platelet agglutinator among AA cascade components. The production of platelet TXA₂ was significantly increased in the DM-0C group relative to the normal group, being consistent with the view of Somova et al (16) that an increment of TXA₂ production in the platelets of STZ-treated rats would be ascribed to pathogenesis of hypertension, cardiac infarction, etc.

Aorta PGI₂ has an incompatible reaction with TXA₂. The production of PGI₂ showed a diminution by 31% in the DM-0C group as compared to that in the normal group, which reflects the increment of platelet aggregative ability in the diabetic state.

The decreased production of PGI₂ in the diabetic state was restored by a dose of catechin and markedly increased in the DM-0.5C and DM-1C groups relative to the DM-0C group, indicating a higher increment than the normal group. The results of our experiment support the fact that the diminution of PGI₂ formation and sensitivity to platelet receptor in the diabetic state results in an imbalance of PGI₂/TXA₂ (7, 21), which ultimately causes various complications of vascular disease (22). Interestingly, the pathogenesis of thrombus was lessened by normalizing.
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these values through the catechin dose.

It is therefore reasonable to conclude that dietary catechin has an anti-thrombus function by suppressing both the activities of PLA₂ and cyclooxygenase, reducing the accumulation of TBARS, and normalizing the ratio of PGI₂/TXA₂.

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