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Inhibition of Platelet Aggregation and Arachidonate Metabolism in Platelets by Procyanidins

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Abstract — The effects of procyanidins on platelet aggregation and arachidonate metabolism in platelets were studied. Nine procyanidins were used in this investigation. Procyanidins B-2-S, EEC and C-1 significantly induced the inhibition of platelet aggregation, and the potency of inhibition was comparable with aspirin. Procyanidin B-2-S was used as a representative of procyanidins for further studies on the effect on arachidonate metabolism. In arachidonate metabolism by fatty acid cyclooxygenase pathway, B-2-S inhibited TXB_2 and HHT formation by intact platelets treated with exogenous arachidonic acid. It also inhibited TXB_2 formation measured by a specific radioimmunoassay when the cells were challenged with calcium ionophore A23187. In cell-free system, B-2-S inhibited both TXB_2 and 12-HETE bioxynthesis in platelet microsome and cytosol, respectively. The inhibitory effect on thromboxane biosynthesis might explain the inhibitory effect of procyanidins on platelet aggregation.

Introduction

Procyanidins, which occur widely in plants of a woody habit, consist almost entirely of 5,7,3′,4′-tetrahydroxy- and 5,7,3′,4′,5′-pentahydroxy-flavan-3-ol units linked through carbon-carbon bonds at C-4. They are of considerable importance because of their biological properties including astringency and enzyme inhibition, which are all related to their ability to bind with proteins. Area II-5-C, a fraction isolated from seeds of *Areca catechu* L., has an antihypertensive effect because of its inhibition of angiotensin-converting enzyme activity (1). Procyanidins are also present in bark of *Salix sieboldiana* (2), stem of *Dioscorea cirrhosa* LOUR (3) and bark of *Kandelia candel* (L) DRUCE (4). In the present study, the effect of several procyanidins on platelet aggregation was investigated. Among the products biosynthesized by platelets that show intimate relevance to vascular homeostasis are metabolites of arachidonic acid. Two pathways are known to metabolize arachidonic acid in platelets. Arachidonic acid is converted to 12-L-hydroxyheptadeca-5,8,10-tri-

enoie acid (HHT) and thromboxanes (TX) by the fatty acid cyclooxygenase pathways, and to 12-L-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) by 12-lipoxygenase and then followed by reduction to 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) by 12-HPETE peroxidase. The effects of procyanidins on arachidonate metabolism in platelets were then analyzed.

Materials and Methods

Chemicals

TXB_2 and all other prostaglandin standards were kindly provided by Ono Pharmaceutical Co., Ltd. Osaka, Japan. Bovine γ-globulin, epinephrine, human globulin, arachidonic acid, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloric acid, calcium ionophore A23187, complete Freud's adjuvant and bovine serum albumin (BSA) were from Sigma Chemical Co., St. Louis, MO. Reduced glutathione (GSH) was purchased from Boehringer Mannheim GmbH, F.G.R. [1-^{14}C]Arachidonic acid (56.6 mCi/mmol) and [5,6,8,9,11,12,14,15(n)-^{3}H]thromboxane B_2 were purchased from Amersham International ple (Bucks, UK). Thin-layer chromatographic plates of silica gel 60, 0.25 mm in thickness, were purchased from E. Merck (Darmstadt, F.R.G.). All reagents not specified above were of analytic grade.

Procyanidins

All the procyanidins used in this study were isolated in our laboratories. Their structure were identified by NMR spectra, and are shown in Figure 1. The abbreviations are as follows. EEC: epicatechin-(4β→8)-catechin, ECE: epicatechin-(4β→8)-catechin-(4α→8)-epicatechin, ECC: epicatechin-(4β→8)-catechin-(4-α→8)-catechin, B-2-S: 4′-benzlthioether of procyanidin B-2, CIN: cinchonain IIb.

Animals

Male New Zealand rabbits with body weight of 3 kg were provided by the Animal Center of National Taiwan University Medical School, Taipei. Male Wistar rats aged 3 months were provided by the Animal Center of National Cheng Kung University Medical Center, Tainan, Taiwan. All animals were maintained on a commercial laboratory feed under a constant light-dark cycle (8:00 a.m. to 8:00 p.m.), and water was provided ad lib.

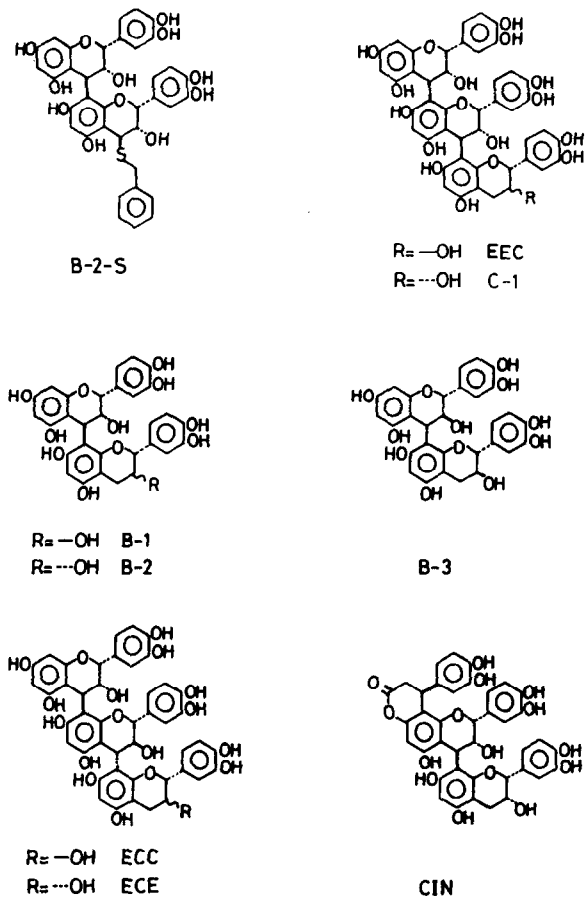

Fig. 1 Structures of procyanidins

Platelet aggregation assay

Blood from human volunteers or rats was withdrawn into a 3.8% solution of sodium citrate (9:1, v/v). Platelet-rich plasma was prepared by centrifugation of citrated blood at 200 × g for 10 min at room temperature. A sample of 0.25 ml of platelet-rich plasma was aggregated at 37°C in a Payton aggregometer with 25 μl of ADP.

Transformation of [^{14}C]arachidonic acid by intact platelets

Platelet-rich plasma was centrifuged at 100× g for 10 min. Platelets were resuspended in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (pH 7.4) containing 5.5 mM glucose. Platelet number was determined with a Coulter Counter. For studying the transformation of [1-^{14}C] arachidonic acid by platelets, each assay tube contained 2 × 10^8 platelets in 1 ml of suspension

and 5 μg of [1-^{14}C]arachidonic acid (0.2 μCi/assay, 5.8 Ci/mol), and was incubated at 37°C for 2 min, unless otherwise stated. The reaction was terminated by quickly adding an appropriate amount of 1 N HCl to bring the pH of the reaction mixture to 3.0. The mixture was then subjected to extraction with 5 ml of ethyl acetate. The resulting organic phase was evaporated to dryness with nitrogen gas. Residues were dissolved in a small aliquot of absolute ethanol and applied to thin-layer chromatographic plates. The plates were developed in a solvent system of chloroform/methanol/acetic acid/water (90:8:11:0.8, v/v). After thin-layer chromatography, the plates were autoradiographed using Fuji X-ray film No. Kx medical. Zones corresponding to the radioactive products were scraped into a scintillation vial for the determination of radioactivity by LKB Rackbeta liquid scintillation counter.

Transformation of endogenous arachidonic acid to thromboxane in platelets

Rat platelets (2×10^8 cells) suspended in 1 ml of Ca^{2+}- and Mg^{2+}-free phosphate-buffer saline (pH 7.4) containing 5.5 mM glucose and 2 mM $CaCl_2$ were challenged with 1 μM calcium ionophore A23187 at 28°C for 3 min. Platelets were spinned down by an airfuge centrifuge. TXB_2 in the supernatant was measured by a specific radioimmunoassay.

Preparation of TXB_2-BSA conjugate

TXB_2 was coupled to BSA according to the procedure of Jaffe et al. (5) for the conjugation of prostaglandins to human serum albumin. TXB_2 (1.8 mg) dissolved in 1.8 ml of 10% ethanol-0.02% aqueous Na_2CO_3 was reacted with 7.2 mg of BSA in the presence of 3.6 mg of 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride at pH 5.5. The mixture was stirred for 20 h at 20°C. The product was exhaustively dialyzed against water and then lyophilized and stored at −20°C.

Production of TXB_2 antiserum

The TXB_2-BSA conjugate (0.5 mg) in 1 ml of saline was emulsified in an equal volume of complete Freud's adjuvant and subcutaneously injected into rabbit. The rabbit received the same dose at two week intervals. Two months after immunization, the animal was bled by venous puncture of the ears. The serum was obtained after precipitating the blood cells by centrifugation at 3000 × g for 20 min.

Radioimmunoassay procedure

Incubation and subsequent separation of the free from the bound form of labeled antigen were carried out at room temperature. Antiserum, labeled antigen, and standards were diluted in the standard radioimmunoassay buffer, 0.05 M Tris-HCl, pH 7.5, containing 0.1% gelatin. The incubation mixture (0.4 ml) contained: 0.2 ml of TXB_2 standards or samples, 0.1 ml of antiserum (final dilution, 1:800), and 0.1 ml of [^{3}H]TXB_2 (approximately 10,000 cpm). The incubation was carried out for 1 h. All samples were run in duplicate in 10 × 75 mm glass tubes. Separation of bound from free antigen was achieved by γ-globulin plus dextran-coated charcoal. The method of preparing γ-globulin and dextran-coated charcoal was as follows. Bovine γ-globulin (0.33 g) was first dissolved in 100 ml of 0.9% dextran in standard assay buffer. Charcoal (3.3 g) was then added, and the solution was stirred for 1 h. Immediately before the addition of charcoal suspension, 1 ml of H_2O was added to each incubation tube for easier pipetting. Each incubation tube was capped with a Luckman LP/3S stopper, and 0.2 ml of the charcoal suspension was withheld in the well. Tubes were inverted five times and allowed to stand for 5 min before centrifugation at 100 × g for 10 min. The supernatant containing the antibody-bound antigen was counted in a LKB Rackbeta liquid scintillation counter.

Preparation of platelet microsome and cytosol

Platelets were suspended in 50 mM Tris-HCl buffer (pH 7.4) and sonicated by a Model W-375 Sonicator (Heat Systems-Ultrasonics, Inc.). The homogenate was centrifuged at 9,000 × g for 20 min, and the resulting supernatant was recentrifuged at 105,000 × g for 1 h by a Beckman L8-80M ultracentrifuge. The resulting pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) and designated as the microsomal fraction. The supernatant was designated as the cytosolic fraction.

Biosynthesis of thromboxane by platelet microsome

Each assay tube contained: arachidomic acid, 5 μg; hemoglobin, 1 μM; epinephrine, 1 mM;

and appropriate amount of microsomal fraction in 1 ml of 50 mM Tris-HCl buffer, pH 7.4. Incubation was carried out at 37°C for 10 min. For background controls, the same incubation was performed except boiled microsome was used. Products were extracted with ethyl acetate as described above. TXB_2 formed by platelet microsome was measured by a specific radioimmunoassay.

Platelet 12-lipoxygenase activity assay

The enzyme activity was assayed according to the method described previously (6). The assay mixture contained 12 μg of [1-^{14}C]arachidonic acid (0.2 μCi/assay) and cytosol fraction in the presence of 1 mM GSH in a final volume of 1 ml of 25 mM Tris-HCl buffer (pH 7.7). Incubation was performed in air at 37 °C for 2 min with shaking. Products were extracted with ethyl acetate as described above. Formation of [1-^{14}C]12-HETE was analyzed by thin-layer chromatography in a solvent system of petroleum ether/diethyl ether/acetic acid (50:50:211, by vol).

Protein determinations

Protein contents were determined by the method of Lowry et al. (7) with bovine serum albumin (fraction V) as a standard.

Results

Radioimmunoassay of TXB_2

Antibodies generated against TXB_2-BSA conjugate were found to be specific for TXB_2 as shown in Figure 2. The cross-reaction of the antibodies with other prostaglandins was at least two orders of magnitude lower than PGE_2. The sensitivity was as good as other prostaglandin radioimmunoassays, and picogram levels could be measured quantitatively.

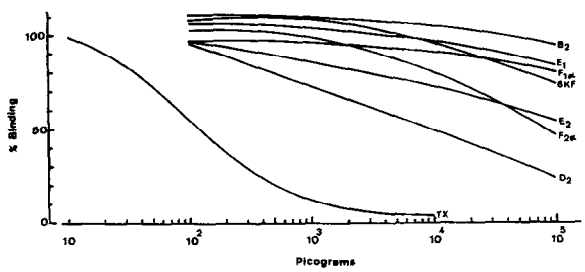

Fig. 2 Specificity of antiserum against TXB_2. TX, D_2, $F_{2\alpha}$, E_2, 6KF, $F_{1\alpha}$, E_1 and B_2 represent TXB_2, PGD_2, $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$, $PGF_{1\alpha}$, PGE_1 and PGB_2, respectively

Inhibition of platelet aggregation by procyanidins

The effect of procyanidins on platelet aggregation was studied by using human and rat platelet-rich plasma, respectively. For comparison, the effect of aspirin was simultaneously tested. As indicated in Table 1, only B-2-S, EEC and C-1 showed a significant inhibition on platelet aggregation in both human and rat platelet-rich plasma. Their inhibitory effect was as comparable as that of aspirin. Since rat platelets were easier to obtain and more reproducible, they were used in the following series of experiments in this study.

Table 1 Inhibition of human and rat platelet aggregation by procyanidins

Compounds	*Inhibition (%)-human*				*Inhibition (%)-rat*	
	10^{-6} M	*10^{-5} M*	*10^{-4}M*	*10^{-3} M*	*10^{-5} M*	*10^{-4} M*
B-2-S	0	5	47	96	12	45
EEC	5	15	60	98	2	58
C-1	2	14	30	98	2	22
B-1	0	0	0			
B-2	1	1	1			
B-3	0	0	2			
ECC	0	0	0			
ECE	12	12	19			
CIN	0	0	16			
Aspirin	0	20	38	42		

Platelet-rich plasma was incubated with the tested compound at 37°C for 30 min. The platelet aggregation of human and rat plasma was then induced by 10^{-5} M and 5 $\times$ 10^{-5} M ADP, respectively. Each value is the mean of triplicate assays.

Effect of procyanidins on arachidonate metabolism in intact platelets

In intact platelet assay system, the kinetics of product formation was somewhat different among TXB_2, HHT and 12-HETE. Figure 3 shows the product formation as function of substrate. Formation of TXB_2 and HHT was maximal at 5 μg of substrate used, while formation of 12-HETE was still linear up to 15 μg of substrate. 5 μg of arachidonic acid was then used in the routine assay. The time course of product formation is shown in Fig. 4. Formation of TXB_2, HHT and 12-HETE was linear up to 2 min incubation. Therefore, 2 min incubation was used in the routine assay. In order to study the effects of procyanidins on arachidonate metabolism in

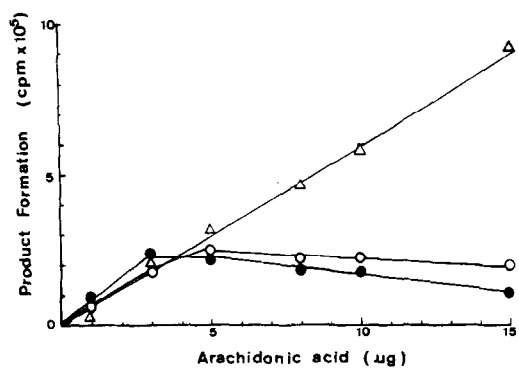

Fig. 3 Formation of arachidonate metabolites in platelets as function of substrate

Assays were performed as described in Methods. Formation of TXB_2 (•), HHT (○) and 12-HETE (△) was measured. Each of points was the mean of duplicate assays

Table 2 Effect of procyanidin B-2-S on the formation of arachidonte metabolites in intact platelets

Groups	*TXB_2*	HHT (cpm)	12-HETE
Control	26594 ± 948 (100%)	33201 ± 2687 (100%)	92360 ± 988 (100%)
10^{-5} M	24258 ± 1248 (91%)	39035 ± 3687 (117%)	83290 ± 1327 (90%)
10^{-4} M	14694 ± 612 (55%)***	25035 ± 1725 (75%)**	80025 ± 720 (86%)**
10^{-3} M	1181 ± 89 (4%)***	2008 ± 172 (6%)***	3506 ± 271 (4%)***

Platelets were treated with procyanidin B-2-S at 37°C for 30 min. Formation of arachidonate metabolites in intact platelets was performed as described in Methods. Values of product formation represent mean ± S.E.M. from 5 assay tubes. Values in parentheses are the percentages compared with their controls. **P < 0.01, ***P < 0.001.

Table 3 Effect of procyanidin EEC on the formation of arachidonate metabolites in intact platelets

Groups	*TXB_2*	*HHT (cpm)*	*12-HETE*
Control	39556 ± 2428 (100%)	53723 ± 7042 (100%)	93595 ± 1222 (100%)
10^{-5} M	37005 ± 1468 (94%)	49394 ± 5528 (92%)	93043 ± 5776 (99%)
10^{-4} M	31416 ± 1875 (79%)**	40774 ± 2401 (76%)	88340 ± 3581 (94%)

Platelets were treated with procyanidin EEC at 37°C for 30 min. Formation of arachidonate metabolites in intact platelets was performed as described in Methods. Values of product formation represent mean ± S.E.M. from 5 assay tubes. Values in parentheses are the percentages compared with their controls.

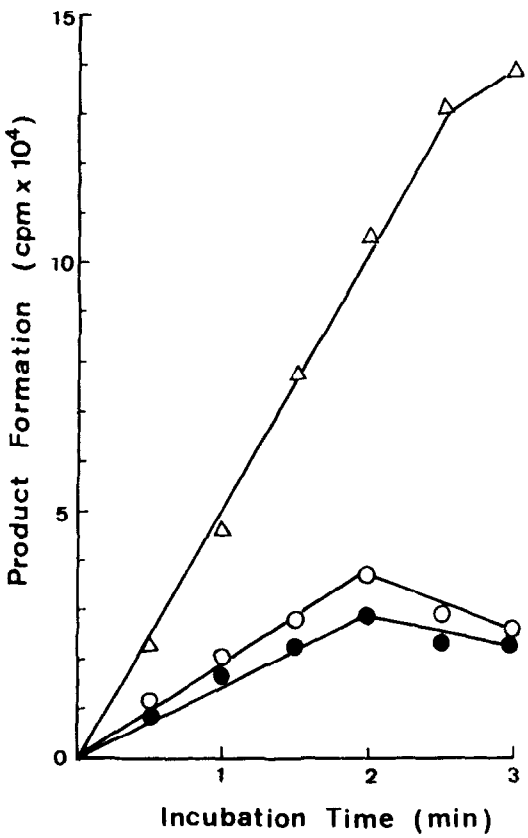

Fig. 4 Time course of product formation in platelets. Assays were performed as function of incubation time. Symbols were the same as described in Fig. 3

intact platelets, cells were pretreated with B-2-S, EEC and C-1 at 37°C for 30 min. The results are summarized in Tables 2–4. Treatment of 10^{-4} M B-2-S induced a significant inhibition on TXB_2, HHT and 12-HETE biosynthesis, and the inhibitory effect was dose-dependent. Both of 10^{-4} M EEC and C-1 also showed an inhibitory effect on TXB_2 biosynthesis while they had less effect on 12-HETE biosynthesis. According to the above results, B-2-S was more potent of inhibiting arachidonate metabolism in intact platelets, and it was therefore studied in the following experiments.

In order to study the effect of B-2-S on thromboxane formation from endogenous arachidonic

Table 4 Effect of procyanidin C-1 on the formation of arachidonate metabolism in intact platelets

Groups	*TXB_2*	*HHT* *(cpm)*	*12-HETE*
Control	29925 ± 950 (100%)	42211 ± 3487 (100%)	135295 ± 2553 (100%)
10^{-5} M	28182 ± 1561 (94%)	39708 ± 1722 (94%)	114020 ± 10123 (84%)
10^{-4} M	24539 ± 801 (82%)	40604 ± 7931 (96%)	116095 ± 7450 (85%)

Platelets were treated with procyanidin C-1 at 37°C for 30 min. Formation of arachidonate metabolites in intact platelets was performed as described in Methods. Values of product formation represent mean ± S.E.M. from 5 assay tubes. Values in parentheses are the percentages compared with their controls.

acid, platelets were challenged with calcium ionophore A23187 after the pretreatment of B-2-S. Biosynthesis of thromboxane in platelets challenged by calcium ionophore A23187 was maximal in 3 min incubation (Fig. 5). Pretreatment of platelets with B-2-S induced a dose-dependent inhibition in thromboxane biosynthesis from the endogenous arachidonic acid (Fig. 6).

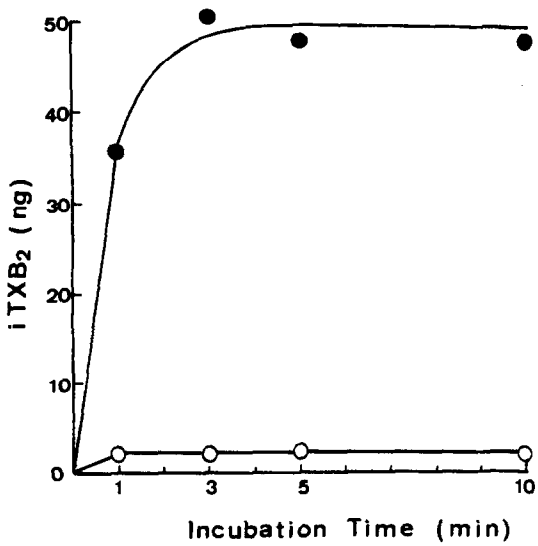

Fig. 5 Thromboxane biosynthesis induced by calcium ionophore A23187 in platelets.

Assays were performed as described in Methods. Closed circles represent cells challenged with calcium ionophore A23187, and open circles represent cells without calcium ionophore challenge. Each of points was the mean of duplicate assays

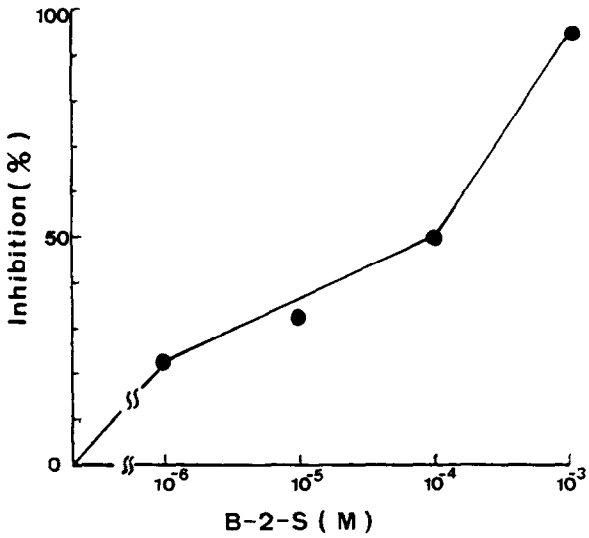

Fig. 6 Inhibition of thromboxane biosynthesis in intact platelets by procyanidin B-2-S.

Platelets were pretreated with procyanidin B-2-S at 37 °C for 30 min, and then treated with calcium ionophore as described in Methods. The thromboxane biosynthesis was compared with control group. Each group was performed in five assays, and the mean of standard error was within 10%

Effect of B-2-S on arachidonate metabolism in platelet cell-free system

Platelet microsomal and cytosolic fractions were used as enzyme source for studying the effects of B-2-S on fatty-acid cyclooxygenase and 12-lipoxygenase pathways, respectively, TXB_2 formation was linear in fashion up to 0.76 mg of microsome used as enzyme source (Fig. 7). B-2-S inhibited the TXB_2 biosynthesis dose-dependently (Fig. 8). In the 12-lipoxygenase pathway, B-2-S dose-dependently inhibited the enzyme activity (Fig. 9), which resulted in decrease in the formation for 12-HETE.

Discussion

Among the procyanidin derivatives tested, B-2-S, EEC and C-1 showed a significant inhibition on platelet aggregation in both human and rat rich plasma (Table 1). B-2-S was chosen as a representative of procyanidins for further studies on the effects on arachidonate metabolism in platelets. Both of fatty acid cyclooxygenase and 12-lipoxygenase pathways were analyzed. In arachidonate metabolism by fatty acid cyclooxygenase pathway, B-2-S inhibited TXB_2 and HHT formation by intact platelets treated with

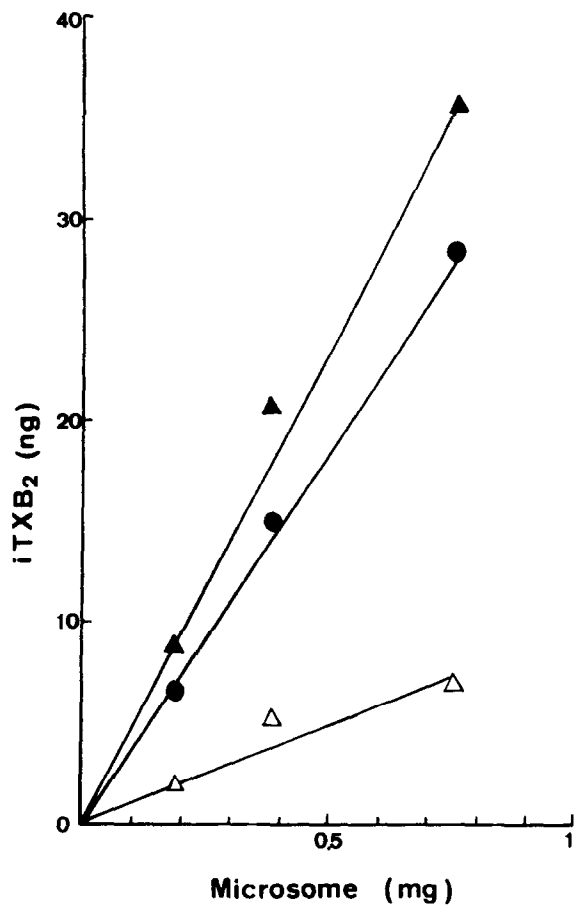

Fig. 7 Thromboxane biosynthesis in platelet microsome.
Assays were performed as described in Methods. The incubation was carried out for 10 min. The net biosynthesis of thromboxane (●) was calculated by substracting the background formed in the absence of arachidonic acid (△) from the immunoreactive TXB_2 formed in the presence of arachidonic acid (▲). Each of points was the mean of five assays

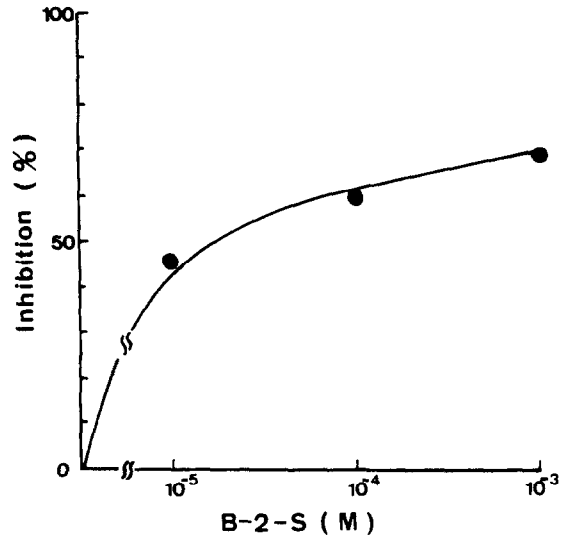

Fig. 8 Inhibition of thromboxane biosynthesis in platelet microsome by procyanidin B-2-S.
Assays were performed in the presence of 0.5 mg of platelet microsome. The thromboxane biosynthesis was compared with control group. Each group was performed in five assays, and the mean of standard error was within 10%

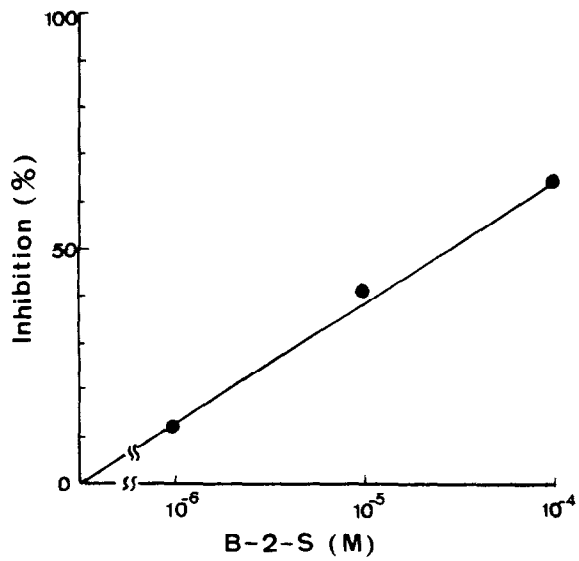

Fig. 9 Inhibition of platelet 12-lipoxygenase activity by procyanidin B-2-S.
Assays were performed in the presence of 0.7 mg of platelet cytosol as described in Methods. The 12-HETE biosynthesis was compared with control group. Each group was performed in quadraplicate assays, and the mean of standard error was within 10%

exogenous arachidonic acid. It also inhibited TXB_2 formation measured by a specific TXB_2 radio-immunoassay developed in our laboratories, when the cells were treated with calcium ionophore A23187 for inducing the endogenous arachidonate release. In cell-free system, B-2-S inhibited TXB_2 formation from arachidonic acid by platelet microsome. Two enzymes; fatty acid cyclooxygenase and thromboxane synthetase, are involved in thromboxane biosynthesis. The results described in the present study indicated that procyanidin B-2-S inhibits fatty acid cyclooxygenase in platelets. Since the experiment with PGH_2 as substrate was not performed,

whether B-2-S inhibits thromboxane synthetase is not clear. In arachidonate metabolism by 12-lipoxygenase pathway, B-2-S inhibited 12-HETE formation by intact platelets treated with exogenous arachidonic acid. The inhibitory effect on 12-HETE formation was confirmed by a cell-free assay. B-2-S inhibited 12-lipoxygenase activity in platelet cytosol. The overall results strongly indicated that procyanidin B-2-S inhibits both fatty acid cyclooxygenase and 12-lipoxygenase pathways in platelets.

Several procyanidins were used in this study. Procyanidins B-1, B-2, C-1 and CIN the bark of Kadelia Candel (L) Druce (4). B-3 and EEC the bark of *Salix sieboldiana* Blume (2). EEC and ECC were from *Dioscrea cirrhosa* Lour (3). B-2-S was from partial acid-catalysed degradation of procyanidin C-1. Procyanidin B-2 and its monomer; (-)-epicatechin, are also present in Linderae umbellatae Ramus, and exhibit the antipeptic activity when administered orally in polorus-ligated mice (8). (-)-Epicatechin treatment in alloxan-induced diabetes of rats increases the pancreatic beta-cell regeneration and significantly lowers the blood sugar (9). In the present study, we found that procyanidin B-2-S, EEC and C-1 inhibits the thromboxane biosynthesis in platelets, which might be able to explain their inhibitory effect on platelet aggregation.

Acknowledgments

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