Inhibitory Effects of Epigallocatechin-3-Gallate on Microsomal Cyclooxygenase-1 Activity in Platelets

Dong-Ha Lee¹, Yun-Jung Kim¹, Hyun-Hong Kim¹, Hyun-Jeong Cho², Jin-Hyeob Ryu¹, Man Hee Rhee³ and Hwa-Jin Park¹*²

¹Department of Biomedical Laboratory Science, College of Biomedical Science and Engineering and Regional Research Center, Inje University, Gimhae 621-749, ²Department of Biomedical Laboratory Science, College of Medical Science, Konyang University, Daejeon 302-718, ³Laboratory of Veterinary Physiology & Signaling, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Republic of Korea

Abstract

In this study, we investigated the effect of (–)-epigallocatechin-3-gallate (EGCG), a major component of green tea catechins from green tea leaves, on activities of cyclooxygenase (COX)-1 and thromboxane synthase (TXAS), thromboxane A₂ (TXA₂) production associated microsomal enzymes. EGCG inhibited COX-1 activity to 96.9%, and TXAS activity to 20% in platelet microsomal fraction having cytochrome c reductase (an endoplasmic reticulum marker enzyme) activity and expressing COX-1 (70 kDa) and TXAS (58 kDa) proteins. The inhibitory ratio of COX-1 to TXAS by EGCG was 4.8. These results mean that EGCG has a stronger selectivity in COX-1 inhibition than TXAS inhibition. In special, a nonsteroid anti-inflammatory drug aspirin, a COX-1 inhibitor, inhibited COX-1 activity by 11.3% at the same concentration (50 μM) as EGCG that inhibited COX-1 activity to 96.9% as compared with that of control. This suggests that EGCG has a stronger effect than that of aspirin on inhibition of COX-1 activity. Accordingly, we demonstrate that EGCG might be used as a crucial tool for a strong negative regulator of COX-1/TXA₂ signaling pathway to inhibit thrombotic disease-associated platelet aggregation.

Key Words: (–)-Epigallocatechin-3-gallate (EGCG), Aspirin, Microsomal fraction, Cyclooxygenase-1, Thromboxane synthase

INTRODUCTION

An important role in the mechanism that agonists induce platelet aggregation is played by an aggregation-inducing molecule thromboxane A₂ (TXA₂) formation (Malmsten et al., 1975; Lewis and Watts, 1982; Li et al., 2010), which can cause circulatory disorders such as thrombosis, atherosclerosis, and myocardial infarction (Schwartz et al., 1990). TXA₂ is generated from arachidonic acid (AA) released when membrane phospholipids are broken down by diverse agonists such as collagen, thrombin, and ADP, and powerfully induces platelet activation and vasoconstriction as an autacoidal action (Hamberg et al., 1975; Samuelsson et al., 1978; Gresele et al., 1991). TXA₂ production associated enzymes are cyclooxygenase (COX)-1 and thromboxane synthase (TXAS), which are located at microsomes (Carey et al., 1982). COX-1 produces prostaglandin (PG)G₂ from substrate AA, TXA₂ produces TXA₂ from PGH₂ that oxidized from PGG₂ by endoperoxidase. Therefore, inhibition of COX-1 or TXAS is very useful to evaluate an antiplatelet effect of any substance or compound. For instance, COX-1 inhibitor aspirin and TXAS inhibitor ozagrel are being used as anti-platelet agents (Patrano, 2001).

A major catechin analogue (–)-epigallocatechin-3-gallate (EGCG, Fig. 2A) from green tea has a galloyl group at the 3’ position of catechin, and is known to have an anti-platelet activity by inhibiting p38 mitogen-activated protein kinase, and extracellular signal-regulated kinase-1/2 (Lill et al., 2003) and by reducing thrombin-induced [Ca²⁺] increase via inhibition of Syk and Lyn activities (Deana et al., 2003). EGCG has also been reported to inhibit collagen-induced phospholipase C-γ₂ activity and TXA₂ production (Jin et al., 2008). With regard to the regulatory effects of EGCG on COX-1 or TXAS activity, it is reported that EGCG failed to inhibit COX-1 and TXAS activity (Jin et al., 2008). In addition, it is known that EGCG did not affect COX-1 expression in human prostate carcinoma cells (Hussain et al., 2005). In this study, however, we clarified and characterized the inhibitory effects of EGCG on COX-1 and TXAS activities by using microsomal enzymes both having cy-
tochrome c reductase activity and expressing COX-1 (70 kDa) and TXAS (58 kDa) proteins. This provides a novel information of EGCG-mediated antiplatelet activity.

MATERIALS AND METHODS

Materials

Collagen was obtained from the Chrono-Log Corporation (Havertown, PA, USA). (−)-Epigallocatechin-3-gallate (EGCG), aspirin, and other reagents were obtained Sigma Chemical Corporation (St. Louis, MO, USA). COX-fluorescent activity assay kit and thromboxane A₂ synthase substrate prostaglandin H₂ were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Cytochrome c reductase (NADPH) assay kit and other reagents were obtained Sigma Chemical Corporation (St. Louis, MO, USA). All other chemicals and reagents used in the present study were of the analytical grade.

Preparation of rat washed platelets

We prepared rat washed platelets to obtain microsomal fraction, a source of COX-1 and TXAS. Blood was collected from Sprague-Dawley (SPD) rats (6-7 weeks, male), and anti-coagulated with acid-citrate-dextrose (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 the 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 5×10⁷ cells/ml. All of the above procedures were carried out at 25°C to avoid platelet aggregation on cooling. The ethics committee for animal experiments of Inje University (Gimhae, Gyungnam, Korea) approved these animal experiments.

Isolation of microsomal fraction

Washed platelets (10⁶ platelets/ml) containing suspension buffer (pH 7.4) with 1% protease inhibitor were sonicated at sensitivity 100% for 20 sec, 1 cycle, and 10 times on ice with buffer (pH 7.4) to a final concentration of 5×10⁷ cells/ml. All of the above procedures were carried out at 25°C to avoid platelet aggregation on cooling. The ethics committee for animal experiments of Inje University (Gimhae, Gyungnam, Korea) approved these animal experiments.

Cytochrome c reductase activity assay

Cytochrome c reductase is a flavoprotein localized in the ER. Cytochrome c reductase activity of the fractions (homogenates, F1-, F2-, and F3-cellular fraction) was identified by cytochrome c reductase (a marker enzyme of ER membrane) (Lagarde et al., 1981) and used as enzyme sources in Western blot as described below.

Western blot analysis of cyclooxygenase-1 (COX-1) and TXA₂ synthase (TXAS)

For the measurement of COX-1 activity, the F2-cellular fraction (microsomal fraction, 20 µg proteins) of platelets was pre-incubated with or without various concentrations of EGCG or aspirin at 37°C for 30 min. COX-1 activity of the treated F2-cellular fraction was assayed with COX-1 fluorescent assay kit (Cayman Chemical Co., Ann Arbor, MI, USA).

Thromboxane A₂ synthase (TXAS) activity

For the measurement of TXAS activity of the F2-cellular fraction (microsomal fraction, 20 µg proteins) was preincubated with or without various concentrations of EGCG or aspirin at 37°C for 5 min. The reaction is initiated by the addition of PGH₂. Incubation is allowed to proceed for 1 min at 37°C. The reactions are terminated by the addition of 1 M citric acid. After neutralization with 1 N NaOH, the amount of TXB₂, a stable metabolite of TXA₂, was determined by using TXB₂ EIA kit according to the procedure described by manufacturer.

Statistical analysis

The experimental results are expressed as the means ± S.E.M. accompanied by the numbers 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 less than 0.05 was considered statistically significant.

RESULTS

Determination of enzyme source of COX-1 and TXAS

In our previous reports (Ok et al., 2012), we reported that the amount of TXA₂ (determined as TXB₂) in intact platelets was 4.0 ± 0.1 ng/10⁶ platelets, and this was markedly increased to 356.1 ± 46.9 ng/10⁶ platelets when platelets were stimulated with collagen (10 µg/ml) (Ok et al., 2012). EGCG (50 µM), however, powerfully reduced TXA₂ production to 89.5% (Ok et al., 2012). These results show that EGCG may inhibit the activity of COX-1 or TXAS to suppress the production of TXA₂ in collagen-induced platelet aggregation. Therefore, to deter-
mine whether the suppression of TXA$_2$ by EGCG was involved in inhibition of COX-1 or TXAS, we needed enzyme sources expressing both COX-1 and TXAS proteins. Therefore, we separated F1-, F2-, and F3-cellular fractions from platelet lysates, and measured the activity of cytochrome c reductase, an endoplasmic reticulum (microsomes) marker enzyme. As shown in Fig. 1A, we found that the F2-cellular fraction has the highest specific activity of cytochrome c reductase. Next, we determined which fraction from platelet lysates expresses both COX-1 and TXAS proteins. As the result, remarkably high expressions of COX-1 (70 kDa) and TXAS (58 kDa) were observed in F2-cellular fraction (Fig. 1B). Because these are accordance with that F2-cellular fraction has the highest activity of cytochrome c reductase, it is thought that F2-cellular fraction is microsomal fraction. This microsomal fraction was used to determine the activity of COX-1 and TXAS.

**Effects of EGCG on COX-1 and TXAS activities**

As shown in Fig. 2B, COX-1 activity in the absence of EGCG (control) was 1.59 ± 0.11 nmol/min/protein-mg. However, EGCG inhibited dose-dependently COX-1 activity, and EGCG at 50 μM inhibited COX-1 activity to 96.9% as compared with that (1.59 ± 0.11 nmol/min/protein-mg) of control (Table 1). TXAS activity in EGCG-untreated control was 0.18 ± 0.01 nmol/min/protein-mg. However, EGCG (50 μM) inhibited TXAS activity to 0.14 ± 0.01 nmol/min/protein-mg. In addition, 11 nM of ozagrel used as a positive control inhibited strongly TXAS activity to 0.14 ± 0.01 nmol/min/protein-mg. In addition, 100 μM of aspirin suppressed control activity (1.59 ± 0.11 nmol/mg-protein/min) to 1.36 ± 0.10 nmol/protein-mg/min (inhibition of 14.5%) (Fig. 4, Table 2). We analyzed the combination effect (Berenbaum, 1989; Gaddum, 1940) of EGCG (50 μM) with aspirin (100 μM) on COX-1 activity inhibition. Basal COX-1 activity (1.59 ± 0.11 nmol/mg-protein/min) was inhibited to 14.5% by aspirin (100 μM) (Table 2). In the presence of both EGCG (50 μM) and aspirin (100 μM), COX-1 activity was inhibited 98.7% as compared with that of basal activity (Table 2), which inhibitory degree (98.7%) is lower than the sum (111.4%) of the inhibitory degree (96.9%) by EGCG (50 μM), and the inhibitory degree (14.5%) by aspirin (100 μM). This means that the combination of EGCG (50 μM) and aspirin (100 μM) has no combination effect on COX-1 activity, but may have antagonistic effect each other. The inhibitory

**Fig. 1.** Determination of enzyme source of cyclooxygenase (COX)-1 and thromboxane A$_2$ synthase (TXAS). (A) Cytochrome c reductase activities in homogenates, F1-, F2-, and F3-cellular fraction. NADPH-cytochrome c reductase, the marker enzyme for dense tubular system in platelets (100 μg of total protein) was assayed as using NADPH cytochrome c reductase kit (Sigma). (B) Western blot analysis of COX-1 and TXAS in homogenates, F1-, F2-, and F3-cellular fraction. 30 μg proteins in homogenates, F1-, F2-, and F3-cellular fraction from platelets were separated by 8% SDS-PAGE, transferred to PVDF membranes, and detected by ECL. H: homogenates; F1: F1-cellular fraction; F2: F2-cellular fraction (microsomal fraction); F3: F3-cellular fraction.

**Fig. 2.** Effects of EGCG on cyclooxygenase (COX)-1 activity. (A) Chemical structure of EGCG from Green tea leaves. (B) Effect of EGCG on COX-1 activity in F2-cellular fraction (microsomal fraction). The platelet F2-cellular fraction (20 μg of protein) was preincubated for 30 min at 37°C with or without various concentration of EGCG. The enzyme activity was measured with COX fluorescent assay kit (Cayman). The data are given as the means ± S.E.M. (n=4). ***p<0.001 compared with preincubated F2-cellular fraction (microsomal fraction) without EGCG.
degree of COX-1 by aspirin was largely beyond the scope of those concentrations (50 to 800 μM) (Fig. 4), IC₅₀ value of aspirin in COX-1 inhibition could not be calculated.

**Effects of EGCG on COX-1 protein expression**

Western blot analyses were carried out to investigate if the inhibition of COX-1 by EGCG or aspirin is resulted from suppression of COX-1 protein (70 kDa) expression. As shown in Fig. 5, the degree of COX-1 protein expression by various concentration (10 to 50 μM) of EGCG, and aspirin (50, 100 μM) were not altered as compared with that of control, without of EGCG or aspirin. These results suggest that the inhibition of COX-1 activity by EGCG or aspirin is independent on reduction in amount of COX-1 protein, COX-1 mRNA.

**DISCUSSION**

TXA₂ is generated from arachidonic acid (AA) released when membrane phospholipids are broken down by diverse agonists such as collagen, thrombin, and ADP (Hamberg et al., 1975; Samuelsson et al., 1978; Gresele et al., 1991). In previous our report (Ok et al., 2012), it was obvious that EGCG elevates cAMP without affecting cGMP to reduce intracellular Ca²⁺, required for the release of TXA₂ precursor AA from platelet membrane phospholipids. EGCG is also known to inhibit collagen-induced phospholipase Cγ₂, activity to reduce intracellular Ca²⁺ mobilization and TXA₂ production (Jin et al., 2008). TXA₂ is a strong agonist on resting platelets as a positive feedback promoter on activated platelets (Ruggeri, 2002; Jennings, 2009), and is also a vasoconstrictor and a bronchoconstrictor (FitzGerald, 1991). Thus, a compound that can inhibit TXA₂ production-associated enzymes (COX-1 or TXAS), TXA₂ production or TXA₂ action has a potential application as an anti-thrombotic agent (Clutton et al., 2001). Phytochemicals such as ginseng saponin (Lee et al., 2012), chlorogenic acid (Cho et al., 2012), caffedymine (Park, 2007) and sanguinarine (Jeng et al., 2007) inhibited TXA₂ production in agonists-stimulated platelets, which were involved in inhibition of COX-1 activity. These previous reports are accord with fact that the inhibition of agonists-induced platelet aggregation is supported by inhibition of COX-1 rather than TXAS (Lewis and Watts, 1982; Jang et al., 2002). This is supported from result that EGCG inhibits potently COX-1 activity to 96.9%, but mildly inhibited TXAS activity to 20% as compared with those of control (Fig. 2B, 3, Table 1). Because these results suggest that inhibition of TXA₂ by EGCG (Ok et al., 2012) is responsible for the suppression of microsomal COX-1 rather than TXAS, it is shown that EGCG may be used as a COX-1 inhibitor. In addition, it is known that EGCG elevated prostaglandin D₂ (PGD₂), an inhibitor of platelet aggregation, even though EGCG did not inhibit COX-1 and TXAS activities in AA-induced platelet aggregation (Jin et al., 2008). Because intracellular PGD₂ is known to elevate cAMP level to inhibit platelet aggregation (Armstrong, 1996), EGCG that only elevates Ca²⁺-antagonistic cAMP level (Ok et al., 2012) may involve also in inhibition of Ca²⁺-mobilization (Jin et al., 2008; Ok et al., 2012), then Ca²⁺-dependent phospholipase Cγ₂ (PLCγ₂) is inhibited by EGCG, which is resulted in inhibition of AA release from membrane phosphatidylinositol 1,4,5-tri-

| Table 1. Inhibitory degree of EGCG in COX-1- and TXAS-activities |
|------------------------|--------|--------|------------------------|
|                        | COX-1  | TXAS   |
|                        | (nmol/protein-mg/min) | (nmol/protein-mg/min) | Inhibition (%) | Inhibition (%) | COX-1 / TXAS |
| Control                | 1.59 ± 0.11 | 0.18 ± 0.01 | 0 | 20 | 0.05 ± 0.04 |
| EGCG (50 μM)          | 0.05 ± 0.04 | 0.14 ± 0.01 | 96.9 | 4.8 ||

Data were from Fig. 2B, and Fig. 3. Inhibition (%) = (Control–EGCG (50 μM)) / Control* 100. COX-1 / TXAS: inhibitory ratio of COX-1 to TXAS by EGCG.

Fig. 3. Effect of EGCG on thromboxane synthase activity The platelet F2-cellular fraction (microsomal fraction, 20 μg proteins) was preincubated for 5 min at 37°C with or without various concentration of EGCG. The reaction was initiated by the addition of 5 μM PGH₂ and terminated by the addition by 1 M citric acid. The activity was reflected by the production of TXB₂, which was determined by TXB₂ EIA kit (GE Healthcare). The data are given as the means ± S.E.M. (n=4). *p<0.05, **p<0.01, ***p<0.001 compared with preincubated F2-cellular fraction without EGCG.

Fig. 4. Effects of aspirin on COX-1 activity. The platelet F2-cellular fraction (microsomal fraction, 20 μg proteins) was preincubated with or without various concentration of aspirin for 30 min at 37°C. The enzyme activity was measured with COX fluorescent assay kit (Cayman). The data are given as the means ± S.E.M. (n=4). *p<0.05, **p<0.01 compared with preincubated F2-cellular fraction without aspirin.
EGCG (μM) - 10 30 50 - Aspirin (μM) - - - - 50 100

COX-1 activity (nmol/protein-mg/min) Inhibitory degree (%)

Basal 1.59 ± 0.11 0
EGCG (50 μM) 0.05 ± 0.004** 96.9
Aspirin (50 μM) 1.41 ± 0.11 11.3
Aspirin (100 μM) 1.36 ± 0.01 14.5
EGCG (50 μM) + Aspirin (100 μM) 0.02 ± 0.01 98.7

The platelet F2-cellular fraction (microsomal fraction, 20 μg proteins) was preincubated for 30 min at 37°C with or without EGCG (50 μM) or aspirin (50, 100 μM). The enzyme activity was measured with COX fluorescent assay kit (Cayman). The data are given as the means ± S.E.M. (n=4). **p<0.01 compared with preincubated F2-cellular fraction without EGCG or aspirin. Inhibitory degree (%) = (Basal – factors) / Basal × 100 (factors; EGCG, aspirin, EGCG + aspirin)

ACKNOWLEDGMENTS

This study was supported by a grant (2012-0002802 to Hwa-Jin Park) from the Basic Science Research Program via the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology, Korea.

REFERENCES


