Novel anti-platelet activity of protocatechuic acid through inhibition of high shear stress-induced platelet aggregation

Keunyoung Kim, Kyung-Min Lim, Ji-Yoon Noh, Seojin Kang, Ka Young Chung,
Ok-Nam Bae*, and Jin-Ho Chung*

College of Pharmacy, Seoul National University, Seoul 151-742, Korea (K.K., K.-M.L., J.-Y.N., S.K., J.-H.C.); School of Pharmacy, Sungkyunkwan Universtiy, Suwon, Gyeonggido 440-746, Korea (K.Y.C.); College of Pharmacy, Hanyang University, Ansan, Gyeonggido 426-791, Korea (O.-N.B.)

* To whom correspondence should be addressed.
A. Running Title: Novel anti-platelet effect of protocatechuic acid

B. Corresponding author:

Ok-Nam Bae, Ph.D., Email: onbae@hanyang.ac.kr, Tel: 82-31-400-5805, Fax: 82-31-400-5958.

C. Number of text pages:

Number of tables: 2

Number of figures: 5 (including one scheme)

Number of references: 58

Number of words in Abstract: 217

Number of words in Introduction: 615

Number of words in Discussion: 1439

D. List of abbreviations:

ACD, acid citrate dextrose; ADP, adenosine diphosphate; FITC, fluorescein isothiocyanate;

GP, glycoprotein; LDH, lactate dehydrogenase; PE, phycoerythrin; PGE\textsubscript{1}, prostaglandin E\textsubscript{1};

PPP, platelet poor plasma; PRP, platelet rich plasma; vWF, von Willebrand factor; WP, washed platelets

E. Recommended section: Cardiovascular
Abstract

Bleeding is the common and serious adverse effect of currently available anti-platelet drugs. Many efforts are being made to develop novel anti-thrombotic agents without bleeding risks. Shear stress-induced platelet aggregation (SIPA) which occurs under abnormally high shear stress plays a crucial role in the development of arterial thrombotic diseases. Here we demonstrated that protocatechuic acid (PCA), a bioactive phytochemical from Lonicera (honeysuckle) flowers, selectively and potently inhibits high shear (>10,000 s⁻¹)-induced platelet aggregation. In isolated human platelets, PCA decreased SIPA and attenuated accompanying platelet activation including intracellular calcium mobilization, granule secretion and adhesion receptor expression. Anti-SIPA effect of PCA was mediated through blockade of von Willebrand factor (vWF) binding to activated GPIb, a primary and initial event for the accomplishment of SIPA. Conspicuously, PCA did not inhibit platelet aggregation induced by other endogenous agonists like collagen, thrombin or ADP that are important both in pathological thrombosis and normal haemostasis. Anti-thrombotic effects of PCA were confirmed in vivo in rat arterial thrombosis model, where PCA significantly delayed the arterial occlusion induced by FeCl₃. Of a particular note, PCA did not increase bleeding time in rat tail trans-section model, while conventional anti-platelet drugs, aspirin and clopidogrel substantially prolonged it. Collectively, these results suggest that PCA may be a novel anti-platelet agent
which can prevent thrombosis without increasing bleeding risks.
Introduction

Under pathological conditions, platelets become rapidly activated and build up a stable haemostatic plug around injured blood vessel wall through forming platelet aggregates (Varga-Szabo et al., 2008). While platelet aggregation and activation is important for normal haemostasis, it also plays crucial roles in the pathogenesis of thrombotic diseases such as coronary artery diseases and ischemic stroke (Davi and Patrono, 2007). Anti-platelet agents like aspirin, clopidogrel and abciximab are proved to be effective in preventing these thrombotic diseases (Jackson and Schoenwaelder, 2003). However, the risk of bleeding such as intracranial hemorrhage and gastrointestinal bleeding also increases since these agents interfere with normal haemostasis (Serebruany et al., 2004; Michelson, 2010). An ideal anti-platelet agent should selectively block pathological thrombosis without affecting normal haemostatic process (Barrett et al., 2008). Enthusiasm for new anti-platelet drugs with better safety and efficacy is ever increasing which has fueled efforts from academy and pharmaceutical companies to identify compounds with novel anti-platelet mechanisms.

Shear stress-induced platelet aggregation (SIPA) is a distinct type of platelet responses which plays a major role in pathological thrombosis. On the other hand, it is weakly involved in normal haemostasis. Shear stress remains low (~ 200 s$^{-1}$) under healthy conditions, but high shear stress (up to 10000 s$^{-1}$) could occur in the abnormal hemodynamic conditions such as
stenotic area, atherosclerosis, cancer or vascular spasm (Kroll et al., 1996; Miyazaki et al., 1996). Elevated levels of shear stress can directly activate platelets and provoke platelet aggregation, which is initiated by a distinctive interaction between von Willebrand factor (vWF) and glycoprotein Ib (GP Ib) (Goto et al., 1998; Ruggeri et al., 1999; Lenting et al., 2010). Intraplatelet signaling pathways are subsequently stimulated, resulting in increase of intracellular calcium level, secretion of active mediators from granules, and expression of adhesion molecules on platelet extracellular membrane, leading to platelet aggregation and thrombus formation (Nesbitt et al., 2009). These events can occur in the absence of exogenously added chemical agonists; therefore, SIPA has been postulated to play a key role in pathological arterial thrombosis while it contributes minimally to normal haemostatic process (Miyazaki et al., 1996; Tsuji et al., 1999; Kulkarni et al., 2000).

Despite the importance of SIPA in thrombosis and a huge potential of anti-SIPA agents as a novel anti-thrombotic drug with better safety profiles, there are few known pharmacological modulators of SIPA. SIPA and its initial event, vWF and GP Ib binding under high shear stress has been one of the most promising targets (Wang et al., 2002; Jackson and Schoenwaelder, 2003; Barrett et al., 2008; Clemetson and Clemetson, 2008). Blockers of vWF binding to GPIb that include peptide (aptamer), protein or antibody drugs are being actively examined for their therapeutic effectiveness and improved safety in terms of bleeding (Gilbert et al., 2007;
Wadanoli et al., 2007; Kiefer and Becker, 2009). However, few studies have published on phytochemicals or herbal medicines that have anti-SIPA or vWF-GP Ib blocking effects.

In this study, we found that extract of Lonicera flowers, a traditional herbal medicine, had inhibitory effects against SIPA. Among bioactive ingredients in Lonicera flowers, protocatechuic acid (PCA), a benzoic acid derivative widely distributed in edible plants and herbal medicines, demonstrated significant anti-SIPA effects in isolated human platelets. Anti-thrombotic efficacy of PCA was confirmed in in vivo arterial thrombosis model. Of note, the effect of PCA was mediated through blocking of vWF-GP Ib interaction, and PCA showed highly selective inhibition against SIPA over other agonist-induced platelet aggregation. Consistently, PCA did not increase bleeding time at effective anti-thrombotic doses in rat tail trans-section model while conventional anti-platelet drugs, aspirin and clopidogrel, substantially prolonged it. With our results, we suggest that PCA has novel therapeutic potential as an anti-platelet agent with reduced bleeding risk.
Materials and Methods

Materials

Protocatechuic acid, thrombin, arachidonic acid, trisodium citrate, HEPES, prostaglandin E₁ (PGE₁), glutaraldehyde, EDTA, EGTA, Tris-base, Tris-HCl, β-NADH, pyruvic acid, Triton X-100, Arg-Gly-Asp, ferric chloride, urethane, aspirin, clopidogrel and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). von Willebrand factor (vWF) was from Calbiochem (San Diego, CA), and collagen, ADP and ristocetin were from Chrono-log Co. (Harvertown, PA). Fluo-3 AM, Pluronic F-127 and Alexa Flour® 488-conjugated fibrinogen were from Invitrogen (Eugene, OR), and ¹⁴C-serotonin (55 mCi/mmol) and ACSII scintillation cocktail was obtained from GE Healthcare (Buckinghamshire, UK). Phycoerythrin (PE)-labeled monoclonal antibody against human CD42b (anti-CD42b-PE Ab), fluorescein (FITC)-labeled anti-CD62P antibody (anti-CD62P-FITC Ab) and FITC-labeled PAC-1 (PAC-1-FITC) were from BD bioscience (San Diego, CA), and FITC-labeled anti-vWF antibody (anti-vWF-FITC) was from Abcam (Cambridge, UK).

Plant materials, extraction and isolation

Lonicera flowers (Lonicera japonica) was from China and authenticated by Prof. J. H. Lee (College of Oriental Medicine, Dongguk University, Gyeongju, Korea). A voucher specimen has been deposited at College of Oriental Medicine, Dongguk University. The powdered Lonicera flowers was refluxed with 70% EtOH for 3 hr at
70-80°C. The ethanol extract was evaporated to dryness under reduced pressure. PCA was isolated from the ethanol extract of Lonicera flowers and its structure was identified as described in Lee et al (2010). The ethanol extract of Lonicera flowers was suspended in H₂O and partitioned with hexane, CH₂Cl₂, EtOAc and BuOH. The ethyl acetate fraction was loaded on a silica gel column and eluted with a gradient of CH₂Cl₂/MeOH/H₂O (70:8:5, 70:10:5, 70:16:5, 70:20:5, 7:3:1 and 13:7:2) to give 16 fractions. The fraction 5 was chromatographed on a silica gel column and eluted with a gradient of hexane/EtOAc (75:25 to 100% in 5% steps) and sub-fraction was purified on an RP-18 column with 50% MeOH to obtain PCA. PCA isolated by this method was used for Fig. 1A, B and C. For other in vitro and in vivo results, PCA purchased from Sigma was used to secure the amount and ensure the quality of the material. We confirmed that isolated and commercial PCA showed similar activities against SIPA inhibition (data not shown). PCA was dissolved in DMSO (final 0.2%) and for in vivo experiments, PCA was dissolved in saline.

Preparation of human platelets With an approval from the Ethics Committee of Health Service Center at Seoul National University, human blood was collected from healthy male volunteers (18-25 years old) who had not taken any medications for at least 14 days. Blood was anticoagulated with 3.8% trisodium citrate solution (1:9) for preparation of platelet rich plasma
(PRP), or with acid-citrate-dextrose (ACD; 85 mM trisodium citrate, 71 mM citric acid, 111 mM glucose, 1:6) in the presence of PGE\textsubscript{1} (1 \textmu M) for preparation of washed platelets (WP). All procedures were conducted at room temperature and the use of glass containers and pipettes was avoided. PRP was prepared by centrifugation for 15 min at 150 g, and platelet poor plasma (PPP) was obtained from the precipitated fraction of PRP by centrifugation for 20 min at 2000 g. The platelet count in PRP was adjusted to $3 \times 10^8$ platelets/mL using PPP. For preparation of WP, PRP was centrifuged for 10 min at 500 g, and platelet pellet was washed in Tyrode buffer (134 mM NaCl, 2.9 mM KCl, 1.0 mM MgCl\textsubscript{2}, 10.0 mM HEPES, 5.0 mM glucose, 12.0 mM NaHCO\textsubscript{3}, 0.34 mM Na\textsubscript{2}HPO\textsubscript{4} and 0.3% BSA, pH 7.4) containing 1 \textmu M PGE\textsubscript{1} and 10% ACD. After centrifugation at 500 g for 10 min, platelets were resuspended with Tyrode buffer, and the cell number was adjusted to $3 \times 10^8$ cells/mL. The final CaCl\textsubscript{2} concentration was adjusted to 2 mM prior to use.

*Measurement of shear-induced platelet aggregation (SIPA)* Platelets were subjected to shear stress at 1,500, 2,700, 5,400 or 10,800 s\(^{-1}\) at 37°C for 3 min using a cone-plate viscometer (RotoVisco 1, HAAKE, Karlsruhe, Germany). For WP, 10 \mu g/mL of vWF was added before applying shear stress (Mistry et al., 2000; Miyazaki et al., 1996). The resultant platelets were obtained and diluted with 0.5% glutaraldehyde. Platelet aggregation was determined by
counting single cells with an optical microscope (CX41, Olympus, Japan). Aggregation rate was calculated as follows, Aggregation (%) = (1-A/A₀) x 100. A is number of single platelets in samples, and A₀ is number of single platelets in unsheared control.

*Measurement of intracellular calcium levels* Intracellular calcium change was determined using fluo-3 AM with flow cytometry as previously described (Philipose et al., 2010). Fluo-3 AM (5 μM) and Pluronic F-127 (0.2%) was loaded to platelets in the presence of PGE₁ (1 μM) for 45 min at 37°C in dark. Then platelets were spun-down by centrifugation at 300 g for 10 min, and resuspended with Tyrode buffer. After incubation with PCA and application of shear stress, platelets were diluted with Tyrode buffer. Platelets were analyzed on the FACSCalibur (BD Biosciences, San Jose, CA) equipped with argon laser (λex 488 nm), and data from 5,000 events were collected and analyzed using CellQuest Pro software (BD Biosciences).

*Measurement of granule secretion* Serotonin secretion was measured using radioactive method, following previous reports with slight modifications (Cifuni et al., 2008; Quinton et al., 2002). PRP was pre-incubated with 0.5 μCi/mL ¹⁴C-serotonin (55 mCi/mmol) for 45 min at 37°C, and ¹⁴C-serotonin loaded WP were prepared form PRP as described above. After incubation with PCA and application of shear stress, the reaction was terminated by the addition of EDTA (final
5 mM). The resultant platelet suspensions were centrifuged at 12,000 g for 1 min, and the supernatant was obtained for determination of $^{14}$C-serotonin secretion. Radioactivity in each sample was measured in Wallac 1409 liquid scintillation counter (Perkin Elmer, Boston, MA), after dilution with ACSII scintillation cocktail. Serotonin secretion was expressed as the percentage of total serotonin content as measured in the supernatant from the cell lysed with 0.3% Triton X-100.

Measurement of P-selectin expression, GP IIb/IIIa activation and fibrinogen binding  After incubation with PCA and application of shear stress, platelets were diluted with Tyrode buffer. Anti-CD62P-FITC Ab was used as a marker for P-selectin expression, while platelets were identified by anti-CD42b-PE Ab. Platelets were incubated with anti-CD62P-FITC Ab and anti-CD42b-PE Ab for 20 min in dark and analyzed on the flow cytometer as described above. GP IIb/IIIa activation was determined with flow cytometry using PAC-1, a GP IIb/IIIa activation specific antibody. After incubation with PCA and application of shear stress, platelets were diluted with Tyrode buffer and platelets were incubated with anti-CD42b-PE and PAC-1-FITC for 20 min in dark. Platelets were analyzed on the flow cytometer as described. To determine fibrinogen binding, Alexa Fluor 488 conjugated fibrinogen was used instead of anti-CD62P-FITC Ab or PAC-1-FITC.
**Determination of platelet cytotoxicity** Leakage of lactate dehydrogenase (LDH) from platelets was measured by spectrophotometric analysis. After incubation with PCA, platelets were centrifuged and 50 μL aliquot of the resulting supernatant underwent LDH assay. Briefly, aliquots were added to 1 mL of Tris-EDTA-NADH buffer (56 mM Tris-base, 5.6 mM EDTA and 0.17 mM β-NADH, pH 7.4) and then pre-incubated for 10 min at 37 °C. After pre-incubation, 100 μL of 14 mM warm pyruvate solution was added and the decrease in absorbance at 339 nm resulting from conversion of NADH to NAD⁺ was measured for LDH release. The extent of cell lysis was expressed as the percent of total enzyme activity obtained from a control incubation lysed with 0.3% Triton X-100.

**Measurement of agonist-induced platelet aggregation** Agonist induced platelet aggregation was determined by turbidometric method using an aggregometer (Chrono-log, Havertown, PA). After incubation with PCA for 3 min at 37°C, WP was loaded on the aggregometer and stimulated with thrombin (0.08-0.12 U/mL), collagen (1-4 g/mL), ADP (10-16 μM), arachidonic acid (100-180 μM) or ristocetin (0.2-0.4 mg/mL) for 6 min. For ristocetin induced platelet aggregation, 10 μg/mL of vWF was added before stimulation. Platelet aggregation was measured by light transmission, with 100% calibrated as the absorbance of Tyrode buffer and 0%
calibrated as the absorbance of WP.

**Determination of vWF binding**  After incubation with PCA and application of shear stress, platelets were diluted with Tyrode buffer. Anti-vWF-FITC Ab was used as a marker for vWF binding, while platelets were identified by anti-CD42b-PE Ab. Platelets were incubated with anti-vWF-FITC Ab and anti-CD42b-PE Ab for 20 min in the dark and analyzed on the flow cytometer as described above. For blockade of GP IIb/IIIa, platelets were preincubated with 2 mM of Arg-Gly-Asp.

**Ex vivo determination of anti-SIPA activity**  Male Sprague-Dawley rats (SamTako Co., Osan, Korea) weighing 250-300 g were used for animal experiments. Before the experiments, animals were acclimated for 1 week, and food and water were provided *ad libitum*. All the protocols were approved by the Ethics Committee of Animal Service Center at Seoul National University. For measurement of *ex vivo* shear induced platelet aggregation, one hour after oral administration of PCA, whole blood was collected from abdominal aorta under anesthesia using anticoagulant of 3.8% trisodium citrate (1:9 citrate/blood, v/v). PRP preparation and shear induced platelet aggregation measurement was done as described above.
In vivo arterial thrombosis model  One hour after oral administration of PCA, ferric chloride induced in vivo arterial thrombosis experiment was done. Rats were anesthetized with urethane (1.25 g/kg, i.p.), and approximately 15 mm of the right carotid artery was exposed and dissected free of nerve and connective tissue. 50% FeCl$_3$ soaked filter paper (1 x 2 mm, Whatman No. 1) was applied to the carotid artery for 10 min. An ultrasonic flow-probe was placed around the arterial segment proximal to the injured site. The flow-probe was connected to a Doppler flowmeter (Transonic Systems, Ithaca, USA) to monitor blood flow. The time needed for occlusion to occur was measured for up to 60 min.

In vivo tail bleeding time measurement  One hour after administration of PCA, aspirin, or clopidogrel, rats were anesthetized with urethane, and rat tail was transected at a site 3 mm proximal to the tip. Blood flowing from the incision was gently blotted with filter paper every 30 seconds. Bleeding time was measured as time elapse until bleeding stopped. When bleeding time lasted longer than 30 min, measurement was stopped and bleeding time was recorded as 30 min.

Determination of protocatechuic acid in rat plasma  One hour after administration of PCA, whole blood was collected from abdominal aorta under anesthesia using anticoagulant of heparin.
Plasma was obtained by centrifugation for 2 min at 12000 g, and the plasma was stored at -70°C until analysis. Standard stock solutions of pro catechuic acid were prepared in methanol at concentration level of 5 mg/mL. Working standard solutions were serially diluted with methanol to obtain concentrations for calibration curve standards. Calibration standards of pro catechuic acid (0.1, 0.2, 0.5, 1.0, 5.0, 10.0 and 20.0 µg/mL) were prepared by spiking appropriate amount of the working standard solutions into a pool of drug-free rat plasma. The standard spiked plasma samples were aliquoted (100 µL) into polypropylene tubes and stored at -20°C until analysis. 100 µL aliquots of plasma samples were extracted with 200 µL of cold acetonitrile in 1.5-mL-polypropylene tubes by vortexing for 5 min and were centrifuged at 14,000 rpm, 4 °C for 10 min. The supernatant was transferred into HPLC vial and 1 µL was injected onto a UPLC system. The chromatographic separation was carried out using ACQUITY UPLC system (Waters Co., Milford, MA). The column was ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 x 100 mm). The column temperature and autosampler tray temperature were 40°C and 10°C, respectively. The mobile phase consisted of 0.1 % phosphoric acid (solvent A) and acetonitrile (solvent B). Gradient elution was as follows: 0 – 0.5 min, 95% A – 5% B; 2.5 min, 85% A – 15% B; 3.0 – 4.0 min, 100% B; 4.1 – 5.5 min, 95% A – 5% B. The flow rate was 0.35 mL/min and injection volume was 1 µL. The detection wavelength was set at 260 nm. We controlled the data acquiring and process with Empower 2 software (Waters Co.).
Statistical analysis  All the data are shown as mean ± SEM and the data were subjected to one-way analysis of variance followed by Duncan’s multiple ranged tests to determine which means were significantly different from the control. Statistical analysis was performed using SPSS software (Chicago, IL). In all cases, p value of < 0.05 was used to determine significance.
Results

Lonicera flowers and its active ingredient, protocatechuic acid, significantly attenuated high shear stress-induced platelet aggregation

To examine the effect of Lonicera flower extract on shear-induced platelet aggregation (SIPA), human platelet rich plasma (PRP) was exposed to the high shear rate of 10,800 s\(^{-1}\). As shown in Fig. 1A, Lonicera flower extract significantly inhibited SIPA in a concentration-dependent manner. To identify the active principle in for anti-SIPA effect of Lonicera flowers, 25 μM of known ingredients was treated and the extent of SIPA was measured. Among eight known active ingredients of Lonicera flowers, protocatechuic acid (PCA) significantly inhibited SIPA (Fig. 1B) in a concentration-dependent manner (Fig. 1C). As shown in Fig. 1D, the inhibitory effect of PCA on SIPA was observed under a broad range of shear stress which can be found in various in vivo arterial or pathological conditions (from 1,500 s\(^{-1}\) to 10,800 s\(^{-1}\)). To investigate the underlying mechanism, a human washed platelet (WP) system was introduced, in which the involvement of plasma protein can be excluded. vWF (10 μg/mL) was added before applying shear stress. PCA inhibited SIPA in human WP in a similar pattern to that observed in human PRP (Fig. 1C). The inhibitory effect of PCA was confirmed by microscopic observation where the formation of platelet aggregate was reduced by PCA treatment (Fig. 1E).
PCA inhibited shear-induced intra-platelet signaling and platelet activation

To investigate how PCA attenuated SIPA, we examined intracellular calcium level, a key molecular mediator for platelet activation (Mazzucato et al., 2002; Nesbitt et al., 2002). Shear stress stimulated the intracellular calcium increase, while it was attenuated by PCA in a concentration-dependent manner (Fig. 2A). After cytosolic calcium increases, shear-induced secretion occurs from dense- and alpha-granules, accelerating further platelet activation and aggregation. Dense granule contains small molecules such as ADP and serotonin, and alpha granule contains protein mediators including von Willebrand factor (vWF) and P-selectin (Jurk and Kehrel, 2005). PCA significantly inhibited shear-induced granular secretion from both granules, as determined by reduced serotonin release and P-selectin expression (Fig. 2B and 2C). Furthermore, PCA significantly attenuated GP IIb/IIIa activation, which plays a pivotal role in formation of stable aggregates in SIPA (Fig. 2D). The extent of fibrinogen binding to GP IIb/IIIa by shear stress was also decreased by PCA (Fig. 2E). The inhibitory effects of PCA on platelet function were not from non-specific cytotoxicity as measured by LDH release (Fig. 2F).

Inhibitory effect of PCA on SIPA was mediated by blocking of interaction between vWF and GP Ib
Shear-induced secretion of ADP from dense granules provokes secondary stimulation of adjacent platelets, amplifying activation and aggregation (Mazzucato et al., 2004; Speich et al., 2008). As shown in Fig. 3A, PCA did not affect ADP-induced platelet aggregation, showing that PCA modulated the upstream pathway of ADP secretion. It is reported that SIPA is initiated by the binding of vWF to GP Ibα receptor on platelet membrane (Goto et al., 1998; Ruggeri et al., 1999). As found in vWF binding assay in flow cytometry, PCA significantly inhibited binding of vWF to platelets (Fig. 3B). The inhibitory effect of PCA on vWF binding retained after GP IIb/IIIa blockade, reflecting that PCA interfered with vWF binding to GP Ib but not GP IIb/IIIa, another vWF binding receptor in platelets (Fig. 3C). As shown in Fig. 3D, PCA did not affect the integrity of GP IIb/IIIa. To confirm if PCA interferes with GP Ib-vWF interaction, we measured platelet aggregation by ristocetin, which induces vWF binding to GP Ib without shear stress stimulation (Dong et al., 2001). Notably, PCA significantly inhibited ristocetin-induced platelet aggregation, in a similar pattern to the inhibitory effect on SIPA (Fig. 3E).

**PCA was highly selective against SIPA**

To evaluate if the anti-platelet effect of PCA is SIPA specific, we measured the effect of PCA on platelet aggregation induced by classical agonists such as collagen, thrombin, ADP and
arachidonic acid. While IC_{50} value of PCA against SIPA was 23.33 ± 1.50 \mu M, PCA did not inhibit platelet activation by chemical stimuli within the concentration range tested, demonstrating a high selectivity of PCA on SIPA (Table 1).

**In vivo anti-thrombotic effects of PCA was observed without increased risk of bleeding**

Prior to the evaluation of *in vivo* significance of anti-platelet effect of PCA using rat models, we confirmed the inhibitory effect of PCA on SIPA in rat PRP. PCA attenuated shear-induced rat platelet aggregation in a similar pattern to that observed with human platelets (Fig. 4A). In addition, SIPA was significantly attenuated in the isolated rat platelets after oral administration of PCA (Fig. 4B). Significant *in vivo* anti-thrombotic effects of PCA were observed in rat arterial thrombosis model, as measured by prolonged occlusion time (Fig. 4C). Table 2 shows the plasma PCA concentration following single oral administration. Next, we addressed the effect of PCA on blood clotting and bleeding time, since the most serious adverse effects of anti-platelet agents are impaired blood clotting and prolonged bleeding time. Administration of PCA did not affect coagulation cascade as determined by activated partial thromboplastin time and prothrombin time (data not shown). While aspirin and clopidogrel, the most commonly used anti-platelet agents, significantly increased bleeding time in rat tail transection model, the administration of PCA had no effect on bleeding time up to 100 mg/kg (Fig.
4D), suggesting that PCA does not increase the risk of bleeding.
Discussion

In this investigation, we have demonstrated that protocatechuic acid (PCA), a bioactive ingredient of Lonicera flowers, shows an excellent anti-platelet and anti-thrombotic activity through the selective inhibition of shear-induced platelet activation and aggregation (Scheme 1). In human platelets, PCA decreased high shear stress-induced platelet activation, by blocking of the interaction between von Willebrand factor (vWF) and platelet receptor GP Ib. Anti-thrombotic activity of PCA was observed in rat arterial thrombosis model, while the risk of bleeding was not increased in tail trans-section model in contrast to aspirin and clopidogrel. With this study, we suggest PCA as the novel anti-platelet agent with reduced bleeding risk, which is one of the most serious adverse effects of other clinical anti-platelet therapy.

PCA (CAS No. 99-50-3; C₇H₆O₄; MW=154.12), also known as 3,4-dihydroxybenzoic acid, is one of the natural phenolic acids which is widely distributed in herbal medicines, fruits and spices (Tomás-Barberán and Clifford, 2000). In a study on dietary burden of the phenolic compounds, the daily intake of PCA in Bavarian population was estimated to be upto 4.17 mg (Radtke et al., 1998). In addition to its abundance in edible plants, PCA can be generated by the inter-conversion of other types of phenolic compounds via the metabolism in intestinal microflora (Vitaglione et al., 2007). PCA shows a relatively low plasma protein binding (20.7 ± 0.09% to human albumin) (Kurlbaum and Hogger, 2011), and several types of PCA metabolites
following methylation, glucuronidation, and glycine conjugation were found in rats after *in vivo* infusion (Cao et al., 2009). It is also known that dehydroxylation and decarboxylation of PCA occur by the gut micro-organisms (Dacre and Williams, 1968).

Based on the epidemiological evidences demonstrating the association between its consumption and the reduction of disease incidence such as cancer and cardiovascular diseases, there has been a growing attention to identify the biological role of PCA (Masella et al., 2012). Chemopreventive potential of PCA were intensively studied in various cancer types including colon, lung and skin. These effects were explained by its anti-oxidant, anti-metastatic and pro-apoptotic activities (Yin et al., 2009; Lin et al., 2011). In the cardiovascular system, PCA is known to reduce oxLDL level (Lee et al., 2002), and to decrease hydrogen peroxide-induced endothelial damage (Chang and Hsu, 1992). Owing to its potent biological activities, PCA is currently suggested as a potential candidate for a pharmaceutical agent, not just as a bioactive component of vegetables and fruits.

Some of the previous studies reported that PCA blocked agonist-induced platelet change such as ADP-, AA-, or collagen-induced platelet aggregation (Yun-Choi et al., 1987; Chang and Hsu, 1992), which was not observed in our present study. The effective concentration of PCA in their study was $10^{-4}$ to $10^{-3}$ M, which is much higher than our experimental system, and this high concentration range might not be easily achieved in *in vivo* system. In contrast, PCA
showed highly selective inhibition on vWF binding and SIPA in a much lower concentration (significant effect at 5 μM; IC₅₀= 23.33 ± 1.50 μM) than other agonist-stimulated phenomena (IC₅₀ > 200 μM) under the same experimental condition. Moreover, the plasma concentration of PCA at the effective anti-thrombotic doses in the rat in vivo model (Table 2) matched well the effective PCA concentration in SIPA inhibition (Fig. 1), suggesting that anti-platelet efficacy of PCA may be mediated by blockade of SIPA, rather than through non-specific inhibition of agonist-induced platelet aggregation.

We measured the concentrations of PCA in rat plasma at one hour after oral administration (Table 2). The plasma concentration of PCA was found to be 8.32 ± 1.64 μM and 28.50 ± 12.13 μM at 10 mg/kg and 25 mg/kg, respectively, where the statistically significant anti-thrombotic effects of PCA were observed (Fig. 4). This concentration range matched well the previously reported pharmacokinetic parameters of PCA in rats. Cmax, Tmax, and T₁/₂ of PCA were reported to be 3.16 ± 0.03 μg/mL, 0.5 ± 0.03 hr, and 3.87 ± 0.25 hr, respectively, following single oral dose of 50 mg/kg PCA (5 g/L solution and 10 mL/kg in the original description) (Han et al., 2007). Herbal extracts such as Danshen also increased blood PCA concentration in rats showing Tmax of 0.5 ± 0.0 hr and T₁/₂ of 0.42 ± 0.01 hr (Zhang et al., 2011).

Up to date, conventional anti-platelet agents target diverse stages of thrombus formation (Jackson and Schoenwaelder, 2003; Barrett et al., 2008). Aspirin inhibits platelet activation by
blocking the synthesis of thromboxane A₂. Clopidogrel and ticlopidine attenuate platelet activation by suppressing ADP signaling via P2Y₁₂ antagonism, while abciximab interferes with the final stage of platelet recruitment and aggregation through the blockade of GP IIb/IIIa. Despite the established efficacy of these anti-platelet agents, bleeding risk also increases during their clinical application (Serebruany et al., 2004; Michelson, 2010). Gastrointestinal hemorrhage is one of the most serious problems of aspirin (Baigent et al., 2009; Sostres and Lanas, 2011). Patients treated with clopidogrel after coronary bypass surgery have significantly increased bleeding problems (Yende and Wunderink, 2001; Chen et al., 2004). In case of GP IIb/IIIa inhibitors, which have been paid special attention due to its potent anti-platelet activity without redundant effects on vascular system, the risk of bleeding significantly increased proportional to the potency of anti-platelet activity (Lefkovits et al., 1995; Scarborough et al., 1999).

To overcome this mechanistically and pharmacologically related adverse effect of anti-platelet agents, a new target or therapeutic approach is required. It is necessary to target the selective pathway that is differently regulated under pathological environment (Barrett et al., 2008). SIPA, which occurs selectively under high shear stress in atherosclerotic lesion, has been suggested as a novel anti-platelet target with a wider margin of safety in terms of bleeding complication (Kroll et al., 1996; Kroll and Feng, 2005). Supporting this view, modulating the
interaction between GP Ib and vWF, the initial and unique event for SIPA, is currently considered as the most attractive target in the development of new anti-platelet agents (Jackson and Schoenwaelder, 2003; Barrett et al., 2008; Clemetson and Clemetson, 2008; Firbas et al., 2010). Here we demonstrated that PCA has potent and selective inhibitory effects against SIPA (Fig. 1), compared to platelet aggregation induced by other chemical agonists (Table 1). These results suggest that PCA may have an anti-platelet activity without increased hemorrhagic risks, which has been demonstrated in vivo in rat arterial thrombosis and in tail trans-section bleeding models (Fig. 5).

In general, high shear stress induces the conformational change of vWF, exposing its A1 domain which interacts with GP Ibα platelet receptor (Miyata et al., 1996; Huizinga et al., 2002; Sadler, 2002). Upon this initial binding, subsequent intra-platelet pathways are activated including intracellular calcium increase, granular secretion of active mediators such as serotonin, ADP or P-selectin, and expression of GP IIb/IIIa. The inhibitory pattern of PCA in each pathway during SIPA is well correlated (Fig. 2A to D), suggesting that PCA might act on the common upstream pathway for these platelet activation pathways. SIPA can be abolished by ADP receptor blockade, since secreted ADP plays a key role in SIPA through second stimulation of ambient platelets (Goto et al., 2002; Mazzucato et al., 2004; Maxwell et al., 2007). As shown by comparing shear- and ADP-induced platelet change, however, PCA affects the
upstream pathway of ADP secretion without affecting ADP-mediated secondary activation pathways (Fig. 3A and B) which conforms well the blockade of vWF binding GP Ib.

Although it remains to be examined how PCA exactly interferes vWF-GP Ib interaction, we can consider three possibilities; i) blocking of GP Ibα receptor, ii) targeting of vWF A1 domain, and iii) inhibition of the conformational change of vWF induced by high shear stress. Since PCA significantly inhibited ristocetin-induced platelet aggregation which occurs in vWF A1- GP Ibα dependent manner in the absence of high shear stress, the third possibility may be excluded. Incidentally, blockers of vWF A1- GP Ibα interaction that have been reported were mostly antibodies or peptide mimetic (Benard et al., 2008; Clemetson and Clemetson, 2008; Kiefer and Becker, 2009). Few studies have published on small molecules that have anti-SIPA or vWF-GP Ib blocking effects, to our best knowledge. Therefore, we propose that the elucidation of the exact mode of action of PCA against vWF-GP Ib interaction may be worthwhile in the field of chemical and structural biology.

In conclusion, we demonstrated anti-platelet and anti-thrombotic effect of PCA, which is mediated by selective and potent inhibitory effects against high shear-induced platelet aggregation through the blockade of vWF-GP Ib interaction. Our results suggest that PCA may represent a novel anti-platelet agent for treatment and prevention of arterial thrombotic complications, with a wide margin of safety for the risk of bleeding problems.
Authorship contributions

- Participated in research design: K.-M.L., O.-N.B. and J.-H.C.
- Conducted experiments: K.K. and J.-Y.N.
- Contributed new reagents or analytic tools: J.-Y.N. and S.K.
- Wrote or contributed to the writing the manuscript: K.-M.L., K.Y.C. and O.-N.B.
References


Mazzucato M, Pradella P, Cozzi MR, De Marco L and Ruggeri ZM (2002) Sequential cytoplasmic calcium signals in a 2-stage platelet activation process induced by the


Serebruany VL, Malinin AI, Eisert RM and Sane DC (2004) Risk of bleeding complications with
antiplatelet agents: meta-analysis of 338,191 patients enrolled in 50 randomized


undergo phosphorylation of Syk at Y525/526 and Y352 in response to pathophysiological

Tomás-Barberán FA and Clifford MN (2000) Dietary hydroxybenzoic acid derivatives – nature,

analysis of mural thrombus formation in various platelet aggregation disorders: distinct


Protocatechuic acid is the major human metabolite of cyanidin-glucosides. *J Nutr* **137**:2043-2048.


This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) [20120000844].
Legends for Figures

Figure 1. Effects of Lonicera flower extract and its ingredients on shear-induced platelet aggregation (SIPA). A to C, After human platelet rich plasma (PRP) was treated with Lonicera flower extract or its ingredients for 3 min, shear-induced platelet aggregation was measured. A, Concentration-dependent inhibitory effects of Lonicera flower extract was shown. B, Inhibitory effects of bioactive ingredients in Lonicera flowers was compared at 25 μM. C, Concentration-dependent inhibitory effects of PCA on SIPA was examined. D, Inhibitory effects of PCA (25 μM) on SIPA at various shear rates was compared. E, The inhibitory effect of PCA on SIPA was measured in human washed platelets (WP). Values are mean ± SEM of 3 independent experiments from different blood donors. * significant differences from control group (p<0.05).

Figure 2. Effects of PCA on shear-induced platelet activation. After human WP was incubated with various concentrations of PCA for 3 min, platelets were exposed to high shear stress. A, Intracellular calcium level was measured using Fluo-3 loaded platelets. B, Serotonin release was measured using 14C-serotonin loaded platelets. C, D and E, P-selectin expression, GP IIb/IIIa activation and fibrinogen binding was measured by flow cytometry. F, Platelet cytotoxicity was determined by leakage of LDH. Values are mean ± SEM of 3
independent experiments from different blood donors. * significant differences from control group (p<0.05).

Figure 3. Effects of PCA on ADP-induced platelet aggregation, shear-induced vWF binding and ristocetin-induced platelet aggregation.  A, After human WP was incubated with various concentrations of PCA for 3 min, platelets was stimulated with ADP (10-16 μM).  B and C, vWF binding was determined by flow cytometry. For blockade of GPIIb/IIIa, platelets were preincubated with Arg-Gly-Asp (2 mM).  D, After human WP was incubated with 25 μM of PCA for 3 min, platelets was stimulated with ADP. GP IIb/IIIa activation and fibrinogen binding was measured by flow cytometry.  E, After human WP was incubated with various concentrations of PCA for 3 min, platelets were stimulated with ristocetin (0.2-0.4 mg/mL). Values are mean ± SEM of 3 to 4 independent experiments from different blood donors. * significant differences from control group (p<0.05).

Figure 4. *In vivo* effects of PCA on thrombus formation and bleeding time in rats.  A, After rat PRP was incubated with various concentrations of PCA for 3 min, shear-induced platelet aggregation was measured.  B, One hour after oral administration of PCA, rat PRP was isolated and ex vivo shear-induced platelet aggregation was examined.  C, One hour after oral
administration of PCA, thrombus formation was determined in FeCl₃-induced rat arterial thrombosis model. After one hour administration of PCA (25 and 100 mg/kg), aspirin (100 mg/kg) and clopidogrel (2.5 mg/kg), the bleeding time was determined in rat tail transection model. Values are mean ± SEM of 3 to 6 animals. * significant differences from control group ($p<0.05$).

Scheme 1. Suggested inhibitory mechanism of PCA on shear-induced platelet aggregation.
### Table 1. Comparison of PCA effect on platelet aggregation by different stimuli.

<table>
<thead>
<tr>
<th>Platelet activator</th>
<th>IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shear stress</td>
<td>23.23 ± 1.50</td>
</tr>
<tr>
<td>Thrombin</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Collagen</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>ADP</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>&gt; 200</td>
</tr>
</tbody>
</table>
Table 2. Plasma concentration of PCA after oral administration.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Conc. (μg/mL) [(μM)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.37 ± 0.10 [2.37 ± 0.63]</td>
</tr>
<tr>
<td>10</td>
<td>1.28 ± 0.25 [8.32 ± 1.64]</td>
</tr>
<tr>
<td>25</td>
<td>4.39 ± 1.87 [28.50 ± 12.13]</td>
</tr>
</tbody>
</table>
**Figure 1**

(A) Aggregation (%) of Lonicera japonica (μg/ml) at different concentrations.

(B) Aggregation (%) of various compounds at different concentrations.

(C) Aggregation (%) of Protocatechuic acid (μM) at different concentrations.

(D) Aggregation (%) at different shear rates (s⁻¹).

(E) Photographs showing control and PCA conditions.
Figure 2
Figure 3

A

**ADP-induced aggregation**

![](image1)

B

**vWF binding (fold of basal)**

![](image2)

C

**GP IIb/IIIa blocked**

![](image3)

D

**ADP-induced activation**

![](image4)

E

**Ristocetin-induced aggregation**

![](image5)
Figure 4

A. In vitro rat platelets

B. Rat ex vivo model

C. Rat in vivo thrombosis model

D. Bleeding time (min)

Protocatechuic acid (µM)

Protocatechuic acid (mg/kg)

Protocatechuic acid (mg/kg)

Control

PCA 25

PCA 100

Aspirin

Clopidogrel

Occlusion time (min)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (%)

Aggregation (
Scheme 1

Shear stress

vWF

Protocatechuic acid

GP Ib

Ca^{2+}

Alpha granule

Dense granule

P-selectin

Serotonin

ADP

GP IIb/IIIa

Ca^{2+}

Shear-induced platelet aggregation