Novel Antiplatelet Activity of Protocatechuic Acid through the Inhibition of High Shear Stress-Induced Platelet Aggregation

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ABSTRACT

Bleeding is the most common and serious adverse effect of currently available antiplatelet drugs. Many efforts are being made to develop novel antithrombotic 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 demonstrate that protocatechuic acid (PCA), a bioactive phytochemical from Lonicera (honesuckle) 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. The anti-SIPA effect of PCA was mediated through blockade of von Willebrand factor binding to activated glycoprotein Ib, 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 in both pathological thrombosis and normal hemostasis. Antithrombotic effects of PCA were confirmed in vivo in a rat arterial thrombosis model, where PCA significantly delayed the arterial occlusion effect. Of particular note, PCA did not increase bleeding times in a rat tail transection model, whereas conventional antiplatelet drugs, aspirin, and clopidogrel substantially prolonged it. Collectively, these results suggest that PCA may be a novel antiplatelet agent that can prevent thrombosis without increasing bleeding risks.

Introduction

Under pathological conditions, platelets become rapidly activated and build up a stable hemostatic plug around injured blood vessel walls by forming platelet aggregates (Varga-Szabo et al., 2008). Although platelet aggregation and activation are important for normal hemostasis, they also play crucial roles in the pathogenesis of thrombotic diseases such as coronary artery diseases and ischemic stroke (Davi and Patrono, 2007). Antiplatelet agents such as aspirin, clopidogrel, and abciximab have been proven 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, because these agents interfere with normal hemostasis (Serebruany et al., 2004; Michelson, 2010). An ideal antiplatelet agent should selectively block pathological thrombosis without affecting normal hemostatic process (Barrett et al., 2008). Enthusiasm for new antiplatelet drugs with better safety and efficacy is increasing and has fueled efforts from academies and pharmaceutical companies to identify compounds with novel antiplatelet mechanisms.

Shear stress-induced platelet aggregation (SIPA) is a distinct type of platelet response that plays a major role in pathological thrombosis. On the other hand, it is weakly involved in normal hemostasis. Shear stress remains low (~200 s⁻¹) under healthy conditions, but high shear stress (up to 10,000 s⁻¹) could occur in 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 (GP) Ib (Goto et al., 1998; Ruggeri et al., 1999; Lenting et al., 2010). Intraplatelet signaling pathways are subsequently stimulated, resulting in an increase of intracellular calcium levels, secretion of active mediators from

ABBREVIATIONS: SIPA, shear stress-induced platelet aggregation; Ab, antibody; AM, acetoxyethyl ester; FITC, fluorescein isothiocyanate; GP, glycoprotein; LDH, lactate dehydrogenase; PCA, protocatechuic acid; PE, phycoerythrin; PGE₁, prostaglandin E₁; PRP, platelet-rich plasma; vWF, von Willebrand factor; WP, washed platelet.
granules, and expression of adhesion molecules on platelet extracellular membranes, 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, whereas it contributes minimally to normal hemostatic processes (Miyazaki et al., 1996; Truji et al., 1999; Kulkarni et al., 2000).

Despite the importance of SIPA in thrombosis and the huge potential of anti-SIPA agents as novel antithrombotic drugs 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, have 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 been published on phytochemicals or herbal medicines that have anti-SIPA or vWF-GP Ib blocking effects.

In this study, we found that the extract of *Lonicera* flowers, a traditional herbal medicine, had inhibitory effects against SIPA. Among the 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. The antithrombotic efficacy of PCA was confirmed in an in vivo arterial thrombosis model. It is noteworthy that 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 antithrombotic doses in a rat tail transection model, whereas conventional antiplatelet drugs, aspirin and clopidogrel, substantially prolonged it. With our results, we suggest that PCA has novel therapeutic potential as an antiplatelet 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 were obtained from Sigma-Aldrich (St. Louis, MO). vWF was from Calbiochem (San Diego, CA), and collagen, ADP, and ristocetin were from Chrono-log (Harverstown, PA). Fluo-3/AM, Fluronic P-127, and Alexa Fluor 488-conjugated fibrinogen were from Invitrogen (Carlsbad, CA), and [14C]serotonin (55 mCi/ mmol) and ACSII scintillation cocktail was obtained from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Phycocerythrin (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 Biosciences (San Jose, CA), and FITC–labeled anti-vWF antibody (anti-vWF-FITC) was from Abcam plc (Cambridge, UK).

**Plant Materials, Extraction, and Isolation.** *Lonicera* flowers (*Lonicera japonica*) were from China and authenticated by Prof. J. H. Lee (College of Oriental Medicine, Dongguk University, Gyeongju, Korea). A voucher specimen has been deposited at the College of Oriental Medicine, Dongguk University. The powdered *Lonicera* flowers were refluxed with 70% EtOH for 3 h at 70 to 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 previously (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. Fraction 5 was chromato-

**Fig. 1.** Effects of *Lonicera* flower extract and its ingredients on SIPA. A of C, after human 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 are shown. B, inhibitory effects of bioactive ingredients in *Lonicera* flowers were compared at 25 μM. C, concentration-dependent inhibitory effects of PCA on SIPA were examined. D, inhibitory effects of PCA (25 μM) on SIPA at various shear rates were compared. E, the inhibitory effect of PCA on SIPA was measured in human WPs. Values are mean ± S.E.M. of three independent experiments from different blood donors. *p < 0.05.
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 dimethyl sulfoxide (final 0.2%), and for in vivo experiments PCA was dissolved in saline.

**Preparation of Human Platelets.** With approval from the Ethics Committee of the 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 the preparation of platelet-rich plasma (PRP) or with acid-citrate-dextrose (95 mM trisodium citrate, 71 mM citric acid, and 111 mM glucose, 1:6) in the presence of PGE\(_2\) (1 \(\mu\)M) for the preparation of washed platelets (WPs). 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 150g, and platelet-poor plasma was obtained from the precipitated fraction of PRP by centrifugation for 20 min at 2000g. The platelet count in PRP was adjusted to 3 \(\times 10^8\) platelets/ml by using platelet-poor plasma. For preparation of WPs, PRP was centrifuged for 10 min at 500g, and platelet pellets were washed in Tyrode buffer (134 mM NaCl, 2.9 mM KCl, 1.0 mM MgCl\(_2\), 10.0 mM HEPES, 5.0 mM glucose, 12.0 mM NaHCO\(_3\), 0.34 mM Na\(_2\)HPO\(_4\), and 0.3% bovine serum albumin, pH 7.4) containing 1 \(\mu\)M PGE\(_2\) and 10% acid-citrate-dextrose. After centrifugation at 500g 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\(_2\) concentration was adjusted to 2 mM before use.

**Measurement of Shear-Induced Platelet Aggregation.** Platelets were subjected to shear stress at 1500, 2700, 5400, or 10,800 s\(^{-1}\)/H\(_9262\) for 3 min at 37°C by using a cone-plate viscometer (RotoVisco 1; Thermo Fisher Scientific, Waltham, MA). For WPs, 10 \(\mu\)g/ml of vWF was added before applying shear stress (Miyazaki et al., 1996; Mistry et al., 2000). 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, Tokyo, Japan). The aggregation rate was calculated as follows: aggregation (\%) = 1 – (A\(_{A0}\)) \times 100, where \(A\) is the number of single platelets in samples, and A\(_{A0}\) is the number of single platelets in unsheared control.

**Measurement of Intracellular Calcium Levels.** Intracellular calcium change was determined by using Fluo-3/AM with flow cytometry as described previously (Philopose et al., 2010). Fluo-3/AM (5 \(\mu\)M) and Fluronic F-127 (0.2%) were loaded to platelets in the presence of PGE\(_2\) (1 \(\mu\)M) for 45 min at 37°C in the dark. Then platelets were spun down by centrifugation at 300g for 10 min and resuspended with Tyrode buffer. After incubation with PCA and the application of shear stress, platelets were diluted with Tyrode buffer. Platelets were analyzed on the FACSCalibur cytometer (BD Biosciences) equipped with an argon laser (\(\lambda\)ex 488 nm), and data from 5000 events were collected and analyzed by using CellQuest Pro software (BD Biosciences).

**Measurement of Granule Secretion.** Serotonin secretion was measured by using the radioactive method, following previous reports with slight modifications (Quinton et al., 2002; Cifuni et al., 2008). PRP was preincubated with 0.5 \(\mu\)Ci/ml [\(^{14}\)C]serotonin (55 mCi/mmol) for 45 min at 37°C, and [\(^{14}\)C]serotonin-loaded WPs were prepared from 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 12,000g for 1 min, and the supernatant was obtained for the determination of [\(^{14}\)C]serotonin secretion. Radioactivity in each sample was measured in a Wallac 1409 liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA) after dilution with an 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 Ib/IIIa Activation, and Fibrinogen Binding.** After incubation with PCA and the application of shear stress, platelets were diluted with Tyrode buffer. Anti-CD62P-FITC Ab was used as a marker for P-selectin expression, whereas 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 the dark and analyzed on the flow cytometer as described above. GP Ib/IIIa activation was determined with flow cytometry by using PAC-1, a GP Ib/IIIa activation-specific antibody. After incubation with PCA and the application of shear stress, platelets were diluted with Tyrode buffer and incubated with anti-CD42b-PE and PAC-1-FITC for 20 min in the dark. Platelets were analyzed on the flow cytometer as described above. 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 \(\mu\)l of aliquot of the resulting supernatant underwent LDH assay. In brief, aliquots were added to 1 ml of Tris-EDTA-NADH buffer (56 mM Tris-base, 5.6 mM EDTA, and 0.17 mM \(\beta\)-NADH, pH 7.4) and then preincubated for 10 min at 37°C. After preincubation, 100 \(\mu\)l of 14 mM warm pyruvate solution was added, and the decrease in absorbance at 339 nm resulting from the conversion of NADH to NAD\(^+\) was measured for LDH release. The extent of cell lysis was expressed as the percentage 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 the turbidometric method using an aggregometer (Chrono-log). After incubation with PCA for 3 min at 37°C, WPs were loaded on the aggregometer and stimulated with thrombin (0.08–0.12 U/ml), collagen (1–4 \(\mu\)g/ml), ADP (10–16 \(\mu\)M), arachidonic acid (100–180 \(\mu\)M), or ristocetin (0.2–0.4 mg/ml) for 6 min. For ristocetin-induced platelet aggregation, 10 \(\mu\)g/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 WPs.

**Determination of vWF Binding.** After incubation with PCA and the application of shear stress, platelets were diluted with Tyrode buffer. Anti-vWF-FITC Ab was used as a marker for vWF binding, whereas 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 the blockade of GP Ib/IIIa, platelets were preincubated with 2 \(\mu\)M Arg-Gly-Asp.

**Ex Vivo Determination of Anti-SIPA Activity.** Male Sprague-Dawley rats (SamTako Co., Osan, Korea), weighing 250 to 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 protocols were approved by the Ethics Committee of the Animal Service Center at Seoul National University. For the measurement of ex vivo shear-induced platelet aggregation, 1 h after oral administration of PCA whole blood was collected from abdominal aorta under anesthesia by using an 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 a 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. Filter paper (1 \(\times\) 2 mm, Whatman, Clifton, NJ) soaked with 50% FeCl\(_3\) 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 Inc., Ithaca, NY) to monitor blood flow. The time needed for occlusion to occur was measured for up to 60 min.

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In Vivo Tail Bleeding Time Measurement. One hour after the administration of PCA, aspirin, or clopidogrel rats were anesthetized with urethane, and their tails were transected at a site 3 mm proximal to the tip. Blood flowing from the incision was gently blotted with filter paper every 30 s. 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 the administration of PCA whole blood was collected from the abdominal aorta under anesthesia by using anticoagulant of heparin. Plasma was obtained by centrifugation for 2 min at 12,000 rpm, and the plasma was stored at −70°C until analysis. Standard stock solutions of protocatechuic acid were prepared in methanol at a concentration level of 5 mg/mL. Working standard solutions were serially diluted with methanol to obtain concentrations for calibration curve standards. Calibration standards of protocatechuic acid (0.1, 0.2, 0.5, 1.0, 5.0, 10.0, and 20.0 µg/mL) were prepared by spiking appropriate amounts 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. Aliquots of plasma samples (100 µl) were extracted with 200 µl of cold acetonitrile in 1.5-ml polypropylene tubes by vortexing for 5 min and centrifuged at 14,000 rpm, 4°C, for 10 min. The supernatant was transferred into HPLC vials, and 1 µl was injected onto a UPLC system. The chromatographic separation was carried out by using an Acquity UPLC system (Waters, Milford, MA). The column was an Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm). The column temperature and autosampler tray temperature were 40 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 to 0.5 min, 95% A and 5% B; 2.5 min, 85% A and 15% B; 3.0 to 4.0 min, 100% B; and 4.1 to 5.5 min, 95% A and 5% B. The flow rate was 0.35 ml/min, and injection volume was 1 µl. The detector wavelength was set at 260 nm. We controlled the data acquiring and process with Empower 2 software (Waters).

Statistical Analysis. All data are shown as mean ± S.E.M. and 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 with SPSS software (SPSS Inc., Chicago, IL). In all cases, p < 0.05 was used to determine significance.

Results

Lonicera Flowers and Their Active Ingredient, Protocatechuic Acid, Significantly Attenuated High Shear Stress-Induced Platelet Aggregation. To examine the effect of Lonicera flower extract on SIPA, human 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 for the 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, 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 vivo arterial or pathological conditions (1500–10,800 s⁻¹). To investigate the underlying mechanism, a human WP system was introduced, in which the involvement of plasma protein could be excluded. vWF (10 µg/ml) was added before applying shear stress. PCA inhibited SIPA in human WPs 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 Intraplatelet Signaling and Platelet Activation. To investigate how PCA attenuated SIPA, we examined the 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, whereas it was attenuated by PCA in a concentration-dependent manner (Fig. 2A). After cytosolic calcium increases, shear-induced secretion occurs from dense granules and α-granules, accelerating further platelet activation and aggregation. Dense granule contains small molecules such as ADP and serotonin, and α-granule contains protein mediators including 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. 2, B and C). Furthermore, PCA significantly attenuated GP Ib/IIa activation, which plays a pivotal role in the formation of stable aggregates in SIPA (Fig. 2D). The extent of fibrinogen binding to GP Ib/IIa by shear stress was also decreased by PCA (Fig. 2E). The inhibitory effects of PCA on platelet function were not from nonspecific cytotoxicity as measured by LDH release (Fig. 2F).
Inhibitory Effect of PCA on SIPA Was Mediated by Blocking the 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 has been 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 the binding of vWF to platelets (Fig. 3B). The inhibitory effect of PCA on vWF binding was 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 whether 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). It is noteworthy that 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 whether the antiplatelet effect of PCA is SIPA specific, we measured the effect of PCA on platelet aggregation induced by classic agonists such as collagen, thrombin, ADP, and arachidonic acid. Although the IC_{50} value of PCA against SIPA was 23.33 ± 1.50 μM, 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 Antithrombotic Effects of PCA Were Observed without Increased Risk of Bleeding. Before the evaluation of the in vivo significance of the antiplatelet 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 antithrombotic effects of PCA were observed in the rat arterial thrombosis model, as measured by prolonged occlusion time (Fig. 4C). Table 2 shows the plasma PCA concentration after single oral administration. Next, we addressed the effect of PCA on blood clotting and bleeding time, because the most serious adverse effects of antiplatelet agents are impaired blood clotting and prolonged bleeding time. The administration of PCA did not affect coagulation cascade as determined by activated partial thromboplastin time and prothrombin time (data not shown). Whereas aspirin and clopidogrel, the most commonly used antiplatelet agents, significantly increased bleeding time in the 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 PCA, a bioactive ingredient of *Lonicera* flowers, shows excellent antiplatelet and antithrombotic 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 the interaction between vWF and platelet receptor GP Ib. Antithrombotic activity of PCA was observed in the rat arterial thrombosis model, whereas the risk of bleeding was not increased in the tail transection model in contrast to aspirin and clopidogrel. With this study, we suggest PCA as a novel antplatelet agent with reduced bleeding risk, which is one of

**TABLE 1** Comparison of PCA effect on platelet aggregation by different stimuli

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<tr>
<th>Platelet Activator</th>
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<td>Shear stress</td>
<td>23.33 ± 1.50</td>
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<tr>
<td>Thrombin</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Collagen</td>
<td>&gt;200</td>
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<tr>
<td>ADP</td>
<td>&gt;200</td>
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<tr>
<td>Arachidonic acid</td>
<td>&gt;200</td>
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the most serious adverse effects of other clinical antiplatelet therapy.

PCA (CAS 99–50-3; C7H6O4; molecular weight 154.12), also known as 3,4-dihydroxybenzoic acid, is one of the natural phenolic acids that is widely distributed in herbal medicines, fruits, and spices (Tomás-Barberán and Clifford, 2000). In a study on the dietary burden of the phenolic compounds, the daily intake of PCA in a Bavarian population was estimated to be up to 4.17 mg (Radtke et al., 1998). In addition to its abundance in edible plants, PCA can be generated by the interconversion 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 Högger, 2011), and several types of PCA metabolites after 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 evidence 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 has been intensively studied in various cancer types including colon, lung, and skin. These effects were explained by its antioxidant, antimetastatic, and proapoptotic activities (Yin et al., 2009; Lin et al., 2011). In the cardiovascular system, PCA is known to reduce oxidized low-density lipoprotein levels (Lee et al., 2002) and 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.

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

We measured the concentrations of PCA in rat plasma at 1 h after oral administration (Table 2). The plasma concentration of PCA was found to be 8.32 ± 1.64 and 28.50 ± 12.13 µM at 10 and 25 mg/kg, respectively, where statistically significant antithrombotic effects of PCA were observed (Fig. 4). This concentration range matched well with the previously reported pharmacokinetic parameters of PCA in rats.

### Table 2

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Plasma Concentration (µg/ml)</th>
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<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>
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<tr>
<td>25</td>
<td>4.39 ± 1.87 (28.50 ± 12.13)</td>
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**Fig. 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, 1 h after oral administration of PCA, rat PRP was isolated, and ex vivo shear-induced platelet aggregation was examined. C, 1 h after oral administration of PCA, thrombus formation was determined in the FeCl₃-induced rat arterial thrombosis model. D, after 1-h administration of PCA (25 and 100 mg/kg), aspirin (100 mg/kg), and clopidogrel (2.5 mg/kg), the bleeding time was determined in the rat tail transection model. Values are mean ± S.E.M. of three to six animals. *p < 0.05.

**Scheme 1.** Suggested inhibitory mechanism of PCA on shear-induced platelet aggregation.
C_{max}, T_{max}, and T_{1/2} of PCA were reported to be 3.16 ± 0.03 μg/ml, 0.5 ± 0.03 h, and 3.87 ± 0.25 h, respectively, after a single oral dose of 50 mg/kg PCA (5 g/liter solution and 10 ml/kg in the original description) (Han et al., 2007). Herbal extracts such as Danshen also have increased blood PCA concentration in rats showing T_{1/2} of 0.5 ± 0.0 h and T_{1/2} of 0.42 ± 0.01 h (Zhang et al., 2011).

Conventional antiplatelet 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 A_2. Clopidogrel and ticlopidine attenuate platelet activation by antagonizing the P2Y_{12} ADP receptor, whereas abciximab interferes with the SIPA through a second stimulation of ambient platelets in response to blocking its GP IIb/IIIa. Despite the established efficacy of these antiplatelet agents, bleeding risk also increases during their clinical application (Serebruany et al., 2004; Michel- son, 2010). Gastrointestinal hemorrhage is one of the most serious problems of aspirin [Antithrombotic Trialists’ (ATT) Collaboration 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 the case of GP IIb/IIIa inhibitors, which have been paid special attention because of their potent antiplatelet activity without redundant effects on the vascular system, the risk of bleeding significantly increased proportionally to the potency of antiplatelet activity (Lefkovits et al., 1995; Scarborough et al., 1999).

To overcome the mechanistically and pharmacologically related adverse effects of antiplatelet agents, a new target or therapeutic approach is required. It is necessary to target the selective pathway that is differently regulated under the pathological environment (Barrett et al., 2008). SIPA, which occurs selectively under high shear stress in atherosclerotic lesions, has been suggested as a novel antiplatelet target with a wider margin of safety in terms of bleeding complications (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 antiplatelet 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 with platelet aggregation induced by other chemical agonists (Table 1). These results suggest that PCA may have an antiplatelet activity without increased hemorrhagic risks, which has been demonstrated in vivo in rat arterial thrombosis and tail transection bleeding models (Fig. 4).

In general, high shear stress induces the conformational change of vWF, exposing its A1 domain, which interacts with the GP Ibα platelet receptor (Miyata et al., 1996; Huizinga et al., 2002; Sadler, 2002). Upon this initial binding, subsequent intraplatelet 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. 2, A–D), suggesting that PCA might act on the common upstream pathway for these platelet activation pathways. SIPA can be abolished by ADP receptor blockade, because secreted ADP plays a key role in SIPA through a 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. 3, A and B), which conforms well with the blockade of vWF binding GP Ib.

Although it remains to be examined how PCA exactly interferes with vWF-GP Ib interaction, we can consider three possibilities: 1) blocking of the GP Ibα receptor, 2) targeting of the vWF A1 domain, and 3) inhibition of the conformational change of vWF induced by high shear stress. Because PCA significantly inhibited ristocetin-induced platelet aggregation, which occurs in a vWF A1-GP Ib-dependent manner in the absence of high shear stress, the third possibility may be excluded. Incidentally, blockers of vWF-GP Ib 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 been published on small molecules that have anti-SIPA or vWF-GP Ib blocking effects to our 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 have demonstrated the antiplatelet and antiatherothrombotic effects 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 antiplatelet agent for the treatment and prevention of arterial thrombotic complications with a wide margin of safety for the risk of bleeding problems.
Novel Antiplatelet Effect of Protocatechuic Acid


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