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Mechanisms of attenuation of clot formation and acute thromboembolism by syringic acid in mice

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ABSTRACT

Several *in vitro* studies have proposed syringic acid as a benzoic acid derivative found in plant foods, which can reduces collagen-stimulated platelet aggregation. Nonetheless, to date, there are no reports elucidating the role of syringic acid in platelet activation and thrombosis. Syringic acid inhibited clot formation and procoagulant protease activity and induced blood clot degradation. Syringic acid decreased the expression of density-enhanced phosphatase-1 (DEP-1)/protein tyrosine phosphatase-1B (PTP1B)/$\alpha_{IIb}\beta_3$, as well as the phosphorylation of kinases in collagen/epinephrine-stimulated platelets both *in vitro* and *in vivo*. Moreover, syringic acid inhibited the secretion of granule constituents, clot retraction, and $FeCl_3$-induced vascular occlusion of the carotid artery. Syringic acid (up to 100 μM) did not show any effect on the viability of RAW 264.7 cells. Our findings demonstrate that syringic acid attenuate the development of thrombosis and thromboembolism by inhibiting fibrin clot formation, coagulant factors, and platelet stimulation through DEP-1/PTP1B/$\alpha_{IIb}\beta_3$/kinases.

1. Introduction

Thrombus formation is promoted by platelets through the phases of adhesion, activation and aggregations (Jackson, Nesbitt, & Kulkarni, 2003). As platelets play a crucial role in primary responses, they are extremely sensitive to extracellular stimuli and respond through various membrane receptors and adhesive proteins such as glycoprotein (GP) Ib-IX-V. Cell-matrix interaction and cell-cell interactions are therefore important for the proper and efficient response to extracellular stimuli (Guidetti, Canobbio, & Torti, 2015). The heterodimer receptor, integrin not only stabilizes platelet adhesion but also transmits bidirectional signals through specific ligand binding. Integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ are the most abundant receptors on the surface of platelets. $\alpha_2\beta_1$ binds with collagen, tenascin, and the proteoglycan decorin, while $\alpha_{IIb}\beta_3$ is a receptor for adhesive proteins such as fibrinogen (FIB), von Willebrand factor (vWF), and fibronectin (Bennett, 2005; Guidetti et al., 2002; Ruggeri, Dent, & Saldivar, 1999; Schaff et al., 2010). These ligand bindings are coupled to a set of intracellular effectors, which participate in outside-in and inside-out signaling pathways. The lipid metabolizing enzyme, phosphatidylinositol 3-kinase (PI3K) is involved in almost all signaling pathways of integrin and platelet activation. Therefore, increasing interest has been focused on specific kinases as potential targets for novel anti-thrombotic therapies, with reduced safety challenges, which is a common problem faced with currently used agents (Guidetti et al., 2015; Ghigo, Morello, Perino, & Hirsch, 2013).

Consumption of diets rich in plant-based products containing bioactive polyphenols and relative metabolites protects against cardiovascular disease (Engelfriet, Hoekstra, Hoogenveen, Buchner, & van Rossum, 2010). Syringic acid and several benzoic acid derivatives have been found in plant foods, including walnut, black olive, grape juice, lettuce, cloves, cinnamon bark, potatoes, cereals, beers, and fortified wines (Tomás-Barberán & Clifford, 2000). Several *in vitro* studies suggested that syringic acid, and human urinary metabolites containing *p*-coumaric acid, catechol, and resorcinol reduces collagen-stimulated platelet aggregation and coagulation activity (Kim, Kang, Shin, & Sohn, 2015; Ostertag et al., 2011). These low molecular weight phenolic acids exhibited an inhibitory effect against platelet aggregation at concentrations between 100 and 816 μM/L (Hubbard, Wolffram, Lovegrove, & Gibbins, 2003; Lill, Voit, Schror, & Weber, 2003). In animal studies and clinical trials, the administration of dietary phenolics at concentrations as high as 3000 mg/kg exhibited no significant toxicity (Harwood et al., 2007; Ruiz et al., 2009). Although the potent *in vitro* anti-platelet and anti-coagulation activities of syringic acid have been reported, its antithrombotic action and the mechanism underlying platelet activation and thrombosis are unknown. Despite the low bioavailability and requirement of relatively high concentration to exhibit significant beneficial effects, the beneficial effects of syringic acid on human health, including antioxidant, anti-inflammatory, anticarcinogenic, antiplatelet and anticoagulant effects, reported in previous studies have led to the consideration of syringic acid as a novel

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therapeutic agent (Ostertag et al., 2011).

We recently investigated the antithrombotic activity of edible plant materials such as *Dendropanax morbifera Léveille* (Araliaceae), *Cudrania tricuspidata* (Carr.) Bureau ex Lavallée (Moraceae), and various compounds from these plants, including flavonoids and polyphenols. Among these substances, we newly elucidated the antiplatelet, and antithrombotic activity of syringic acid. Syringic acid, 3,5-dimethoxy-4-hydroxybenzoic acid, is a naturally occurring polyphenol, which is abundant in the solid medium of cultured *Lentinus edodes* mycelia and the second most abundant compound found in a wine made from *Rubus coreanus* (Korean black raspberry) (Forrester et al., 1990; Kim et al., 2015). It is known that syringic acid has antimicrobial, anti-cancer and anti-DNA oxidation effects (Aziz, Faraq, Mousa, & Abo-Zaid, 1998; Guimarães et al., 2007; Kampa et al., 2004). In addition, recent studies have shown its potential hepatoprotective effects and anti-thrombotic effects. At the site of liver injury, syringic acid suppresses inflammatory cytokines, inhibits nuclear factor kappa B (NF-κB)-dependent process and scavenges reactive oxygen species and free radicals (Itoh et al., 2009, 2010). Furthermore, *in vitro* anti-thrombotic effects, including anti-coagulant activity, and anti-platelet aggregation, have been reported (Kim et al., 2015; Kim, Shin, Kang, & Sohn, 2016).

Therefore, this study was designed to investigate the mechanism of action of syringic acid against thrombogenesis and platelet activation, and the potential of syringic acid as a novel antithrombotic and anti-platelet agent. The effects of syringic acid on fibrin clot formation, and procoagulant proteinase activity were investigated. Notably, the anti-platelet effects were examined by measuring collagen/epinephrine (C/E)-stimulated platelet activation. Besides *in vitro* assays, C/E induced acute thromboembolism models and $FeCl_3$-induced arterial thrombosis models were used. The activity of kinases, such as protein kinase B (AKT), PI3K, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and P38, as well as integrin $\alpha_{IIb}\beta_3$ and its ligand FIB were investigated. Furthermore, the expression of anti-platelet targets, density-enhanced phosphatase-1 (DEP-1), protein tyrosine phosphatase-1B (PTP1B), and adenosine monophosphate-activated protein kinase α (AMPKα) were investigated. These results suggest that syringic acid might serve as a potential therapeutic agent owing to its ability to attenuate critical steps by its anticlotting activity and via the signaling target, DEP-1/PTP1B/$\alpha_{IIb}\beta_3$/kinases pathway, involved in the development of thrombosis and thromboembolism.

2. Methods

2.1. Animals

Animals were used for *in vivo* thrombosis model experiments. Male Imprinting control region (ICR) mice (20–30 g; 6 week-old) were used for C/E-induced acute thromboembolism model experiment to evaluate the *in vivo* antithrombotic effects of syringic acid. Male Sprague-Dawley (SD) rats (220–300 g; 8 week-old) were used to establish $FeCl_3$-induced arterial thrombosis model to evaluate the *in vivo* antithrombotic effects. Four mice were housed per cage and kept under controlled conditions of 12 h light/dark cycle and temperature of 22 ± 2 °C. They received a normal diet (Certified Rodent Diet 5002, Orient Bio, Seongnam, Korea) and water ad libitum during the acclimatization and experiment periods. All possible efforts were made to minimize animal suffering and the number of animals used. The whole process was performed according to the 'NIH Guide for the Care and Use of Laboratory Animals' and approved by the Ethics Committee of Chosun University (CIACUC2015-A0017).

2.2. Blood collection and preparation of washed platelets

To analyze the effects of syringic acid on *in vitro* and *in vivo* platelet activity and specific signaling pathways, 2 mL of blood samples were drawn from healthy rodents into 3.8% (w/v) sodium citrate. To prepare murine plasma and platelets, blood was collected from anesthetized mice (6–8 weeks old) and treated with 3% of 75 mM sodium citrate, 39 mM citric acid, 135 mM dextrose (ACD, pH 6.5; 1/9, v/v), and 25 U/mL heparin, and then centrifuged at 190*g* for 5 min, after which platelet-rich plasma (PRP) was collected. PRP was diluted 3-fold in ACD and centrifuged for 10 min at 800*g*. The platelet pellet was suspended in Tyrode's buffer [137 mM NaCl, 12 mM $NaHCO_3$, 2 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 5.5 mM glucose, 5 mM HEPES, and 0.35% bovine serum albumin (BSA)] at a concentration of 250×10^6/mL platelets. $CaCl_2$ (2 mM) was added prior to thrombin or C/E stimulation (0.25 U/mL or 15 mM/10 mM).

2.3. Turbidity assay

Thrombosis is associated with blood clotting and fibrin clotting. Fibrin is formed from fibrinogen by thrombin and is degraded by plasmin and tissue-type plasminogen activator (t-PA). To evaluate the *in vitro* effects of syringic acid on fibrin clot formation, the inhibition of fibrin clot formation was determined by a turbidity assay as previously described (Choi, Sapkota, Park, Kim, & Kim, 2013). The turbidity assay was performed in 96-well plates at room temperature (RT). The reaction mixtures were initially prepared by the addition of syringic acid (5, 10, and 20 μg) or t-PA (5 U) in a volume of 100 μL to fibrinogen (2.9 mM), thrombin (0.5 U/mL) and $CaCl_2$ (5 mM) in 20 mM Tris-HCl (pH 7.4). The resulting mixtures were incubated for 5 min at 37 °C. Immediately after incubation, turbidity was monitored every 1 min at 405 nm for 60 min in a microplate reader (Molecular Devices, Sunnyvale, CA, USA). After the reaction, clot density was expressed as the percentage of inhibition.

2.4. Fibrin clot assay

Fibrin clot assay was performed to investigate the *in vitro* improvement of blood circulation by inhibition of fibrin formation. 90 μL of 1.5% fibrinogen, thrombin (0.5 U/mL) and $CaCl_2$ (5 mM) in 20 mM Tris-HCl were added to different concentrations of syringic acid or 5 U t-PA in a volume of 100 μL. These prepared fibrin clot solutions were incubated at 37 °C for 30 min. Each tube of fibrin clot solution was photographed and weighted. By using the measured weights of each tube, the inhibitory activity was calculated as below:

$$Inhibitoryactivity(\%) = [1-(Weight_{controlwithoutsample} - - Weight_{sample}) / Weight_{controlwithoutsample}]x100$$

Fibrin clots were prepared by mixing human fibrinogen (2.6 μM) and Alexa Fluor 488 fibrinogen (0.4 μM) clotted with 0.25 U/mL of thrombin with syringic acid (5, 10, and 20 μg), or t-PA (10 U) in 20 mM Tris-HCl (pH 7.4) in a glass-bottomed dish. The resulting mixtures were incubated in the dark at 37 °C for 30 min. The fibrin clots were observed with a fluorescent microscope (Nikon, Eclipse TE 2000-U, Japan) and photographed. Saline pre-treated fibrin clots were used as control samples. Fibrin clots pre-treated with t-PA were used as positive control samples. Quantitative evaluation of the fluorescent fibrin clots was performed using ImageJ 1.46b image analysis software (National Institutes of Health, Bethesda, MD, USA).

2.5. In vitro antithrombotic activity

A blood clot is formed by the binding of fibrin (fibrinogen), platelet, and blood cells. To assess the effect of syringic acid on blood clots, we treated syringic acid into the blood clot. Blood clot assay was performed based on a previously described method (Choi, Park, Kim, & Kim, 2015). Tubes containing 40 μg of fresh mouse blood clots were prepared and incubated for 1 h after the addition of syringic acid (10, 20, 50, and 100 μg), t-PA (10, and 20 U), or saline. After incubation, blood clot degradation at each concentration was determined by the absorbance

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