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Inhibition of collagen-induced platelet aggregation by Sanggenon N *via* the Ca^{2+} signaling pathway

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Abstract *Cudrania tricuspidata* (*C. tricuspidata*), a medicinal plant widely employed throughout Asia in ethnomedicine, has various bioactive properties, including antidiabetic, antiobesity, antitumor, and anti-inflammatory activities. In addition, the *C. tricuspidata* root extract reportedly inhibits platelet aggregation. Therefore, we focused on the active substances present in the *C. tricuspidata* extract. Sanggenon N (SN) is a flavonoid found in the root bark of *C. tricuspidata*. In the present study, we examined the inhibitory effects of SN on platelet aggregation, phosphoproteins, thromboxane A_2 generation, and integrin αIIbβ3 activity. SN inhibited collagen-induced human platelet aggregation in a dose-dependent manner without cytotoxicity. Furthermore, SN suppressed Ca^{2+} mobilization and influx through associated signaling molecules, such as inositol 1, 4, 5-triphosphate receptor I (Ser^{1756}), and extracellular signal-regulated kinase. In addition, SN inhibited thromboxane A_2 generation and associated signaling molecules, including cytosolic phospholipase A_2 and mitogen-activated protein kinase p38. Finally, SN could inhibit integrin ($\alpha IIb/\beta_3$) activity by regulating vasodilator-stimulated phosphoprotein and Akt. Collectively, SN possesses potent antiplatelet effects and is a potential therapeutic drug candidate to prevent platelet-related thrombosis and cardiovascular disease.

Keywords Ca^{2+} influx · Ca^{2+} mobilization · *Cudrania tricuspidata* · Sanggenon N

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Introduction

Cardiovascular disease (CVD), known to impact the heart and blood vessels, is the leading cause of death worldwide [1]. CVD includes various diseases, such as atherosclerosis, coronary heart disease, heart attack, heart failure, and stroke. Among the 18.6 million CVD-related deaths recorded worldwide in 2019, an estimated 58% occurred in Asia [2]. The Korean Society of Cardiology has recently revealed that CVD-related mortality has increased in Korea over the last decade [3]. Several studies are underway in Korea to investigate the relationship between cardiovascular disease and other diseases or distinct lifestyles. Among several risk factors of CVD, platelet-mediated thrombosis is considered one of the most important, and various antiplatelet drugs are used to treat cardiovascular disease [4]. Under physiological conditions, vascular endothelial cells release nitric oxide to prevent platelet activation and coagulation cascade. However, following the occurrence of a vascular endothelial injury, platelets adhere to the exposed collagen fiber on the vascular endothelial wall, and platelet-platelet interaction is initiated to form a hemostasis plug. After the interaction of the collagen fiber with binding molecules on the platelet membrane surface, signaling molecules within platelets are activated. Among the various signaling mechanisms, calcium is an important molecule involved in platelet activation. Calcium mobilization is initiated by the breakdown of phosphatidylinositol 4,5-bisphosphate mediated via phospholipase $C\gamma_2$. After decomposition, inositol 1,4,5-trisphosphate (IP_3) binds to the IP_3 receptor (IP_3R) on the endoplasmic reticulum surface, and Ca^{2+} is mobilized from the endoplasmic reticulum [5]. Simultaneously, the depletion of Ca^{2+} levels in the endoplasmic reticulum causes Ca^{2+} influx, which is regulated via extracellular signal-regulated kinase (ERK) [6]. Elevated $[Ca^{2+}]_i$ levels facilitate α- and δ-granule release (e.g., serotonin, ATP, and ADP), and activated platelets synthesize thromboxane A_2 (TXA_2) through cyclooxygenase-1, hydroxy-peroxidase, and thromboxane synthase [7]. These signaling events, called inside-out signaling pathways, induce conformational changes

in glycoprotein IIb/IIIa (integrin αIIb/β_3), leading to αIIb/β_3-mediated signaling events called outside-in signaling pathways [8].

Cudrania tricuspidata (*C. tricuspidata*) is a tree found in Eastern Asia that has been widely used in traditional Chinese medicine [9]. The roots and leaves of *C. tricuspidata* contain various pharmaceutical substances, comprising 158 flavonoids and 99 xanthones [9] and these chemicals reportedly exhibit various physiological activities [9]; however, reports on substances exerting antiplatelet activity are limited. Accordingly, we evaluated the antiplatelet effects of Sanggenon N (SN).

Materials and Methods

Materials

Human platelets were obtained from the Korean Red Cross Blood Center (Suwon, Korea), and SN was purchased from ChemFaces (Wuhan, China). Antibodies (p38, IP_3R, VASP, ERK, PI_3K, Akt, cytosolic phospholipase A_2, and β-actin) were purchased from Cell Signaling Technology (Danvers, MA, USA). Collagen was obtained from Chrono-Log (Havertown, PA, USA). A serotonin ELISA kit was purchased from Labor Diagnostika Nord GmbH and Co. (Nordhorn, Germany). A thromboxane B_2 assay kit and Fura 2-acetoxymethyl (Fura 2-AM) and fibrinogen (Alexa Fluor 488-conjugated) were obtained from Invitrogen (Eugene, OR, USA).

Human platelet aggregation assay

Briefly, washed human platelets (10^8/mL) were resuspended in the suspension buffer. The platelet suspension was adjusted to a concentration of 10^8/mL, as described previously [16]. SN was dissolved in 0.1% dimethyl sulfoxide, and platelet suspensions (10^8/mL) were pre-incubated for 5 min with SN (50, 75, 100, and 150 μM). Next, collagen was used as an agonist to induce platelet aggregation, which was measured for 5 min. The light transmittance of the platelet suspension was converted to the aggregation rate (%) using an aggregometer (Chrono-Log; Havertown, PA, USA).

Cytotoxicity analysis

Briefly, washed human platelets (10^8/mL) were pre-incubated for 5 min at 37 °C with SN (50, 75, 100, and 150 μM) and centrifuged at 12,000×g to separate the supernatant. Lactate dehydrogenase was detected using an ELISA reader (TECAN, Salzburg, Austria). Platelets completely lysed with triton X100 were used as positive controls.

Ca^{2+} mobilization and influx analysis

Briefly, washed human platelets (10^8/mL) were pre-incubated with Fura 2-AM (5 μM), and platelet-rich plasma was washed with washing buffer and adjusted to a concentration of 10^8/mL. Platelets (10^8/mL) were pre-incubated with SN (50, 75, 100, 150 μM) for 5 min at 37 °C and stimulated with collagen (2.5 μg/mL) for Ca^{2+} mobilization. Next, we investigated Ca^{2+} influx. Platelets (10^8/mL) were pre-incubated with SN (50, 75, 100, and 150 μM) in the presence of 100 μM EGTA, stimulated with thapsigargin (1 μM), and 2 mM Ca^{2+} was added at 3 min. Ca^{2+} analysis was performed using a fluorescence spectrophotometer (F-2700; Hitachi, Japan). $[Ca^{2+}]_i$ values were calculated using the Grynkiewicz method [10].

Western blotting assay

After collagen-stimulated platelet aggregation with SN (50, 75, 100, and 150 μM), protein in the platelet lysate was measured using a BCA protein assay kit and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, proteins on the membrane were incubated with primary antibodies (4 °C, overnight) and secondary antibodies (2 h, room temperature). Detection was performed in a dark room, and bands were converted into graphs using the Quantity One program (Bio-Rad, Hercules, CA, USA).

Measurement of ATP and serotonin release

Platelet aggregation was conducted in the presence of SN for 7 min at 37 °C, and the reaction cuvette was placed on ice for 3 min to terminate the release action. After termination, the reaction mixture was centrifuged, and the supernatant was collected. ATP and serotonin levels were detected using an ELISA reader (TECAN).

Conformational changes of αIIb/β_3

To examine αIIb/β_3 activation, platelet aggregation was performed using fibrinogen (Alexa Fluor 488-fibrinogen dye). Following collagen-stimulated platelet aggregation with SN (50, 75, 100, and 150 μM) for 3 min, reaction tubes were incubated with fibrinogen dye for 20 min and fixed with 0.5% paraformaldehyde. The fixed mixture was filtered through a mesh in a flow cytometry tube, and the binding force was analyzed using flow cytometry (BD Biosciences, San Jose, CA, USA).

Thromboxane B_2 (TXB_2) analysis

After collagen-stimulated platelet aggregation with SN (50, 75, 100, and 150 μM), indomethacin (0.2 mM) was added to terminate the conversion. TXA_2 is quickly converted to TXB_2; therefore, we measured TXB_2 generation. The reaction tubes were centrifuged to obtain the supernatant, which was assessed using an ELISA reader according to the manufacturer's instructions (Tecan).

Data analysis

Data values are presented as mean ± standard deviation for various numbers of observations. To determine major differences among groups, analysis of variance was performed, followed by the Tukey-Kramer method. SPSS 21.0.0.0 (IBM Corp., Armonk, NY, USA) was used to perform statistical analysis, and $p < 0.05$ was considered statistically significant.

Results

SN inhibits platelet aggregation and cell cytotoxicity

To analyze the SN-induced platelet aggregation rate (Fig. 1A), collagen (2.5 μg/mL) was used as an agonist, establishing a collagen-stimulated human platelet aggregation rate of 88%. However, SN inhibited collagen-induced platelet aggregation (Fig. 1B). Furthermore, SN-treated platelets showed no cytotoxicity (Fig. 1C).

SN blocks $[Ca^{2+}]_i$ levels, inositol 1, 4, 5-triphosphate receptor type I (IP_3RI)-, ERK phosphorylation, and serotonin release

Next, we evaluated intracellular calcium concentrations and calcium-associated signaling molecules. As shown in Fig. 3A, intracellular calcium levels ($[Ca^{2+}]_i$) were elevated to 642.5±8.5 nM by collagen (2.5 μg/mL). However, SN suppressed elevated $[Ca^{2+}]_i$ levels in a dose-dependent manner (Fig. 2A). It is well-known that IP_3RI, present on the surface of the endoplasmic reticulum, is an important receptor for Ca^{2+} regulation; however, its phosphorylation by cAMP/cGMP-dependent kinases suppresses $[Ca^{2+}]_i$ mobilization [11]. As shown in Fig. 2B, SN enhanced collagen-induced IP_3R phosphorylation. Along with IP_3R-mediated $[Ca^{2+}]_i$ mobilization, Ca^{2+} influx is an important mechanism capable of regulating calcium. The depletion of intracellular Ca^{2+} levels in the endoplasmic reticulum triggers Ca^{2+} influx, and this mechanism is regulated by ERK [6]. Herein, SN inhibited thapsigargin-induced Ca^{2+} influx *via* ERK phosphorylation (Fig. 2C, 2D).

SN blocks serotonin release and ATP release

Next, we examined whether SN can impact ATP and serotonin release in δ-granules. Elevated $[Ca^{2+}]_i$ levels facilitate δ-granule and α-granule release, and ERK is involved in platelet secretion [12]. Accordingly, we evaluated ATP and serotonin release in δ-granules. As shown in Fig. 3A and 3B, SN inhibited collagen-stimulated ATP and serotonin secretion in a dose-dependent manner.

SN blocks fibrinogen binding and VASP, Akt phosphorylation

$αIIb/β_3$ is essential for platelet-platelet interaction and can bind to fibrinogen; thus, we determined whether SN-treated platelets can adhere to fluorescent dye-fibrinogen. As shown in Fig. 4A, collagen could induce fibrinogen binding to $αIIb/β_3$, exhibiting a binding rate of 90.2±2.5% (Fig. 4A-b). However, SN significantly decreased the binding activity (Fig. 4A-c, 4B). Next, we investigated the signaling molecules involved in integrin $αIIb/β_3$ activity. VASP and PI_3K/Akt signaling pathways are the most crucial factors in platelets, leading to adhesive function, platelet spreading, and $αIIb/β_3$ activation [13,14]. Based on our findings, SN significantly downregulated PI_3K/Akt phosphorylation (Fig. 4C, 4D) and upregulated VASP (Ser^{157}) phosphorylation (Fig. 4E).

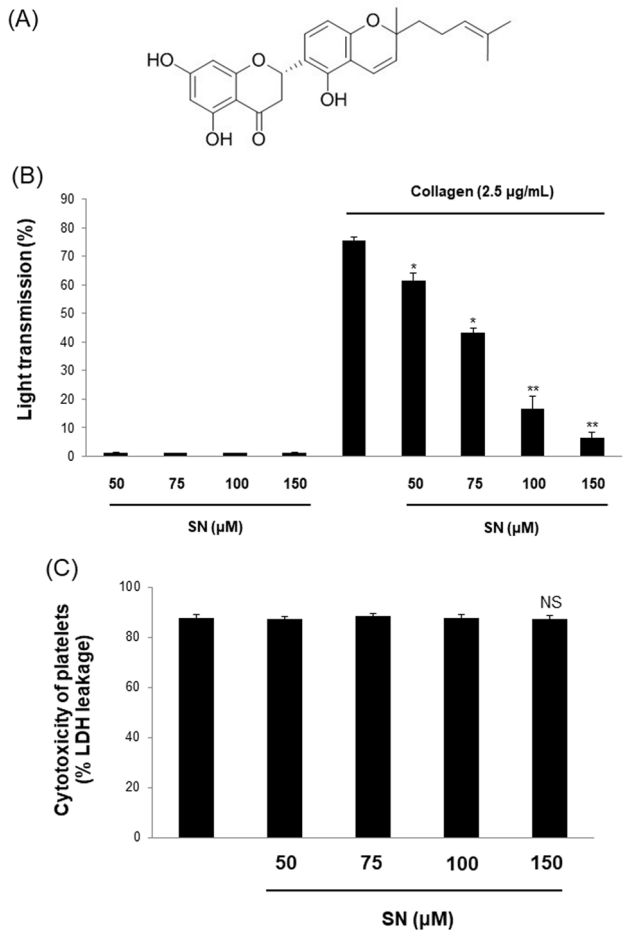

Fig. 1 SN's effect on platelet aggregation. (A) Chemical structure of SN (Sanggenon N, MW. 422.5). (B) SN's effect on collagen-induced human platelet aggregation. (C) SN's effect on cytotoxicity. Platelet aggregation and cytotoxicity were carried out as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n =4). *p <0.05, **p <0.01 versus each collagen-stimulated human platelets. NS, not significant

SN blocks thromboxane B_2 production, and dephosphorylation of $cPLA_2$ and $p38^{MAPK}$

TXA_2 may play a role in the pathogenesis of CVDs [15]. As shown in Fig. 5A, SN inhibited collagen (2.5 μg/mL)-stimulated TXA_2 production. Next, we investigated the signaling molecules involved in TXA_2 production. $cPLA_2$ and p38 are signaling molecules that play critical roles in the synthesis of TXA_2 [16]. Therefore, we evaluated whether SN could affect these signaling molecules. As shown in Fig. 5B and 5C, SN suppressed $cPLA_2$ and p38 phosphorylation in a dose-dependent manner. Finally, the synthesized TXA_2 is secreted from platelets and acts as an aggregating agent for resting platelets. Therefore, we examined whether SN induced an inhibitory effect on U46619 (TXA_2 analog; as an agonist)-induced aggregation. We observed that SN suppressed U46619-induced platelet aggregation in a dose-dependent manner.

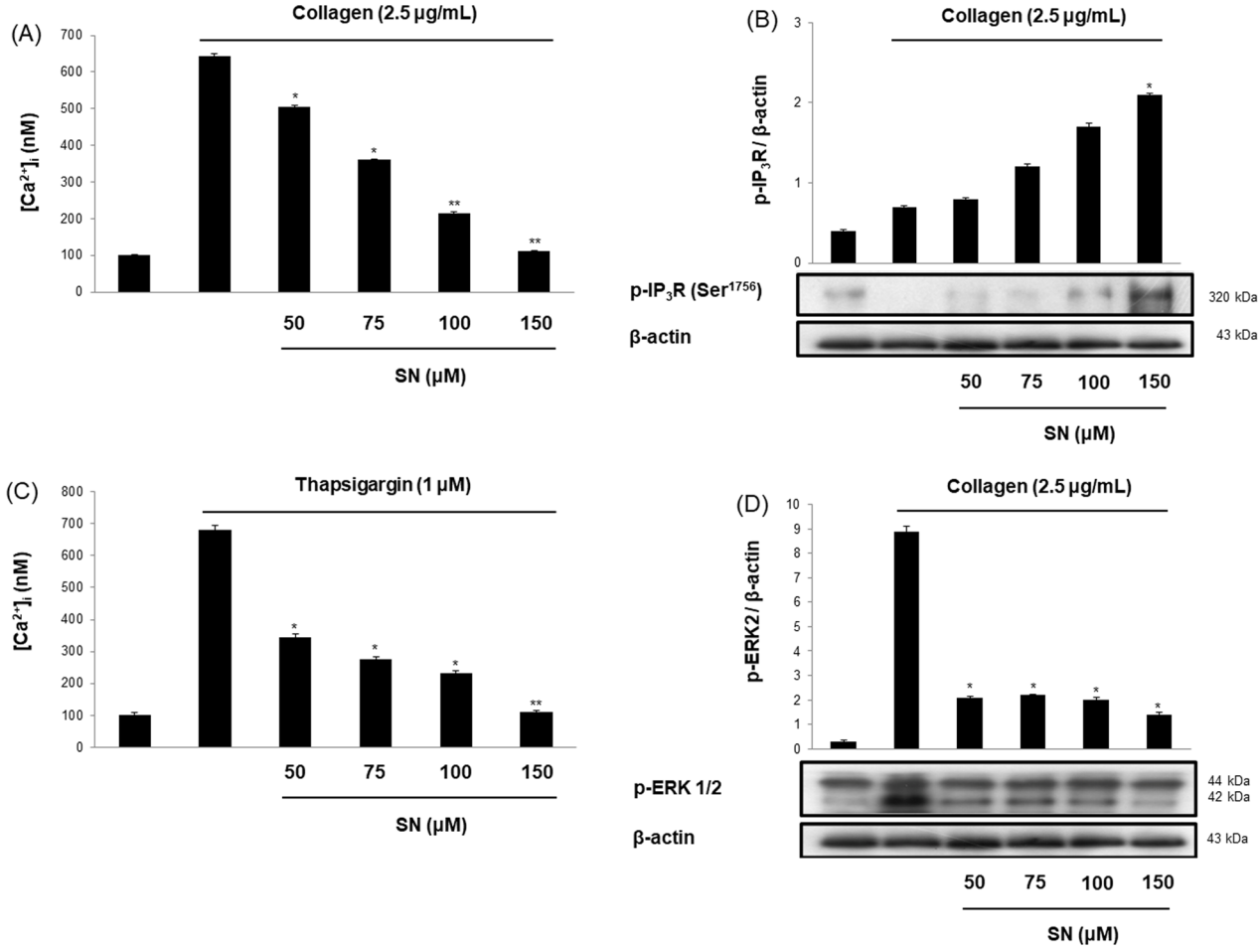

Fig. 2 SN's effect on $[Ca^{2+}]_i$ mobilization, Ca^{2+} influx, IP_3R and ERK phosphorylation. (A) Effect of SN's effect on collagen-induced $[Ca^{2+}]_i$ mobilization. (B) SN's effect on collagen-induced IP_3R phosphorylation. (C) SN's effect on thapsigargin-induced Ca^{2+} influx. (D) SN's effect on collagen-induced ERK phosphorylation. All experiments were performed as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n =4). *p <0.05, **p <0.01 versus the collagen-stimulated human platelets

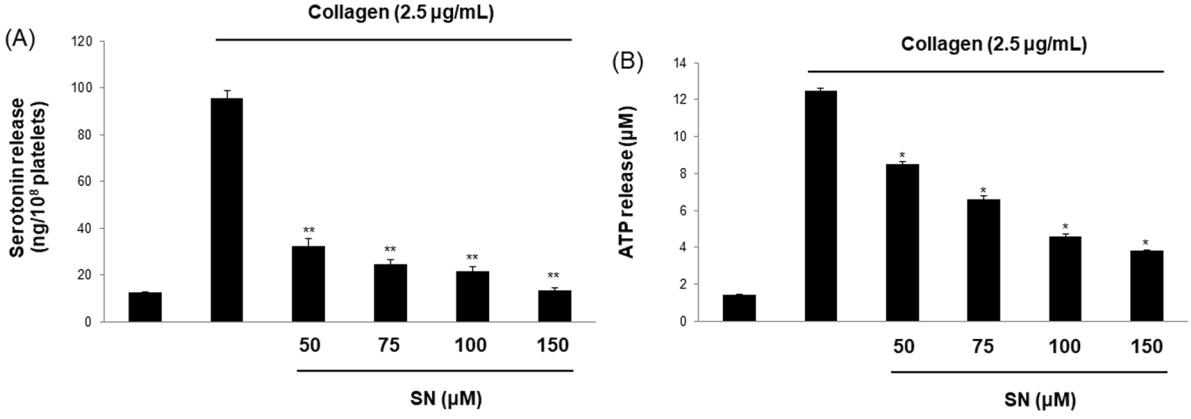

Fig. 3 SN's effect on serotonin and ATP release. (A) SN's effect on serotonin release. (B) SN's effect on ATP release. All experiments were performed as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n =4). *p <0.05, **p <0.01 versus the collagen-stimulated human platelets

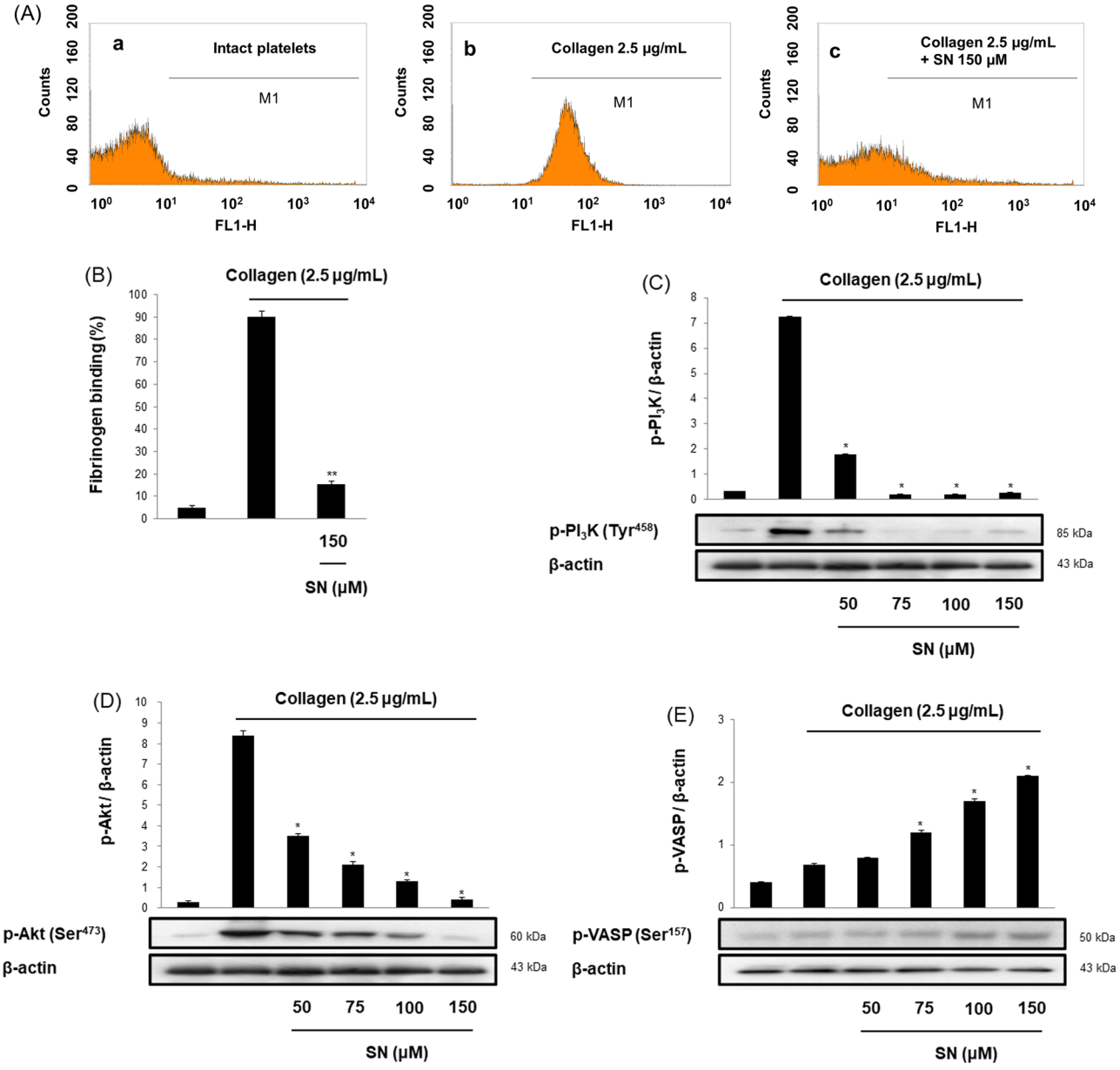

Fig. 4 SN's effect on fibrinogen binding to αIIb/β_3, PI_3K/Akt/VASP phosphorylation. (A) The flow cytometry histograms on fibrinogen binding. (B) SN's effect on collagen-induced fibrinogen binding (%). (C) SN's effect on collagen-induced PI_3K (Tyr^{458}) phosphorylation. (D) SN's effect on collagen-induced Akt (Ser^{473}) phosphorylation. (E) SN's effect on collagen-induced VASP (Ser^{157}) phosphorylation. All experiments were performed as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n =4). *p <0.05, **p <0.01 versus the collagen-stimulated human platelets

Discussion

Changes in $[Ca^{2+}]_i$ are central to platelet activation. Elevated Ca^{2+} leads to the myosin light chain phosphorylation in platelets, and the phosphorylated myosin light chain triggers granule release (i.e., dense body and α-granule) [17]. IP_3R, which controls calcium mobilization, is regulated by phosphorylation. The phosphorylation of IP_3R involves the inhibition of $[Ca^{2+}]_i$ mobilization [18]. Conversely, ERK and Src kinases are well-known mechanisms related to Ca^{2+} influx signaling [19]. We have previously revealed that an ERK inhibitor can suppress Ca^{2+} influx [20]. Therefore, we focused on Ca^{2+} regulation and Ca^{2+} regulatory signaling molecules. Ca^{2+} influx is another Ca^{2+} regulatory mechanism. Stromal interaction molecule 1 ($STIM_1$) has been identified as a key element of the store-operated Ca^{2+} entry mechanism [21, 22]. $STIM_1$ recognizes Ca^{2+} depletion in the endoplasmic reticulum and regulates Ca^{2+} influx. However, the mechanism underlying store-operated Ca^{2+} entry remains debatable.

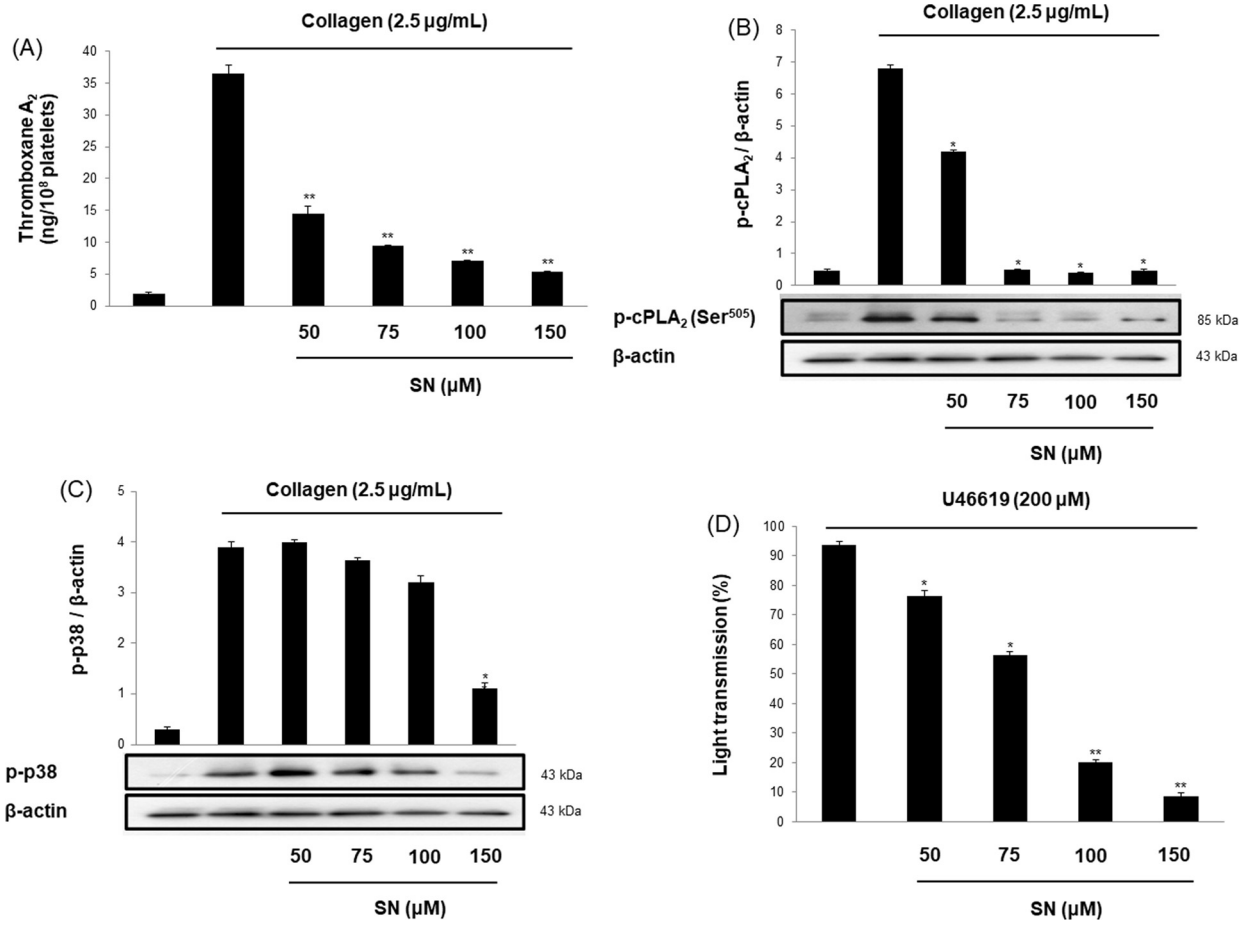

Fig. 5 SN's effect on on TXA_2 production, $cPLA_2$, $p38^{MAPK}$ phosphorylation. (A) SN's effect on collagen-induced TXA_2 generation. (B) SN's effect on collagen-induced $cPLA_2$ phosphorylation. (C) SN's effect on collagen-induced $p38^{MAPK}$ phosphorylation. (D) SN's effect on U46619-induced platelet aggregation. All experiments were performed as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n =4). *p <0.05, **p <0.01 versus the agonists-stimulated human platelets

In the present study, SN could dose-dependently suppress collagen-stimulated human Ca^{2+} mobilization and Ca^{2+} influx (Fig. 2A, 2C). Next, we investigated whether SN inhibited $[Ca^{2+}]_i$ levels by phosphorylating IP_3R (Fig. 2B) and dephosphorylating ERK (Fig. 2D). We confirmed that SN could markedly suppress $[Ca^{2+}]_i$ levels by regulating phosphoproteins. In addition, we examined whether SN affected δ-granule release. As shown in Fig. 3A, 3B, SN inhibited collagen-stimulated ATP and serotonin secretion dose-dependently.

Next, we examined αIIb/β_3 activation. Agonists can stimulate signaling cascades called inside-out signaling, which facilitates integrin activation, leading to structural changes in αIIb/β_3. This conformational change activates αIIb/β_3, which activates a new signaling pathway called outside-in signaling, known to promote platelet aggregation. In addition, αIIb/β_3 can form a hemostatic plug by interacting with other platelets; however, overactivation of this mechanism can result in the generation of blood clots, leading to CVD [23,24]. Therefore, we analyzed signaling molecules that contribute to αIIb/β_3 activation and the effect of SN on the αIIb/β_3-mediated thrombogenic response. Considering the activation of αIIb/β_3, Akt, and VASP, which are the most important mediators, we determined whether SN can suppress αIIb/β_3 action by PI_3K/Akt and VASP. Herein, we observed that SN suppressed αIIb/β_3 affinity (Fig. 4A, 4B), decreased PI_3K/Akt phosphorylation (Fig. 4C, 4D), and increased VASP phosphorylation (Fig. 4E).

Next, we determined whether SN inhibited TXA_2 production (Fig. 5A). TXA_2 is synthesized and secreted by platelets and acts as a potent platelet activator. Signaling molecules related to the generation of TXA_2, $cPLA_2$, and p38 are well established. In the present study, we confirmed that SN suppressed TXA_2 production; hence, we next analyzed the phosphorylation of $cPLA_2$ and p38 in collagen-stimulated human platelets. As shown in Fig. 5B and 5C, SN could inhibit collagen-induced phosphorylation of $cPLA_2$ and p38. In addition, SN suppressed U46619-induced platelet aggregation in a dose-dependent manner.

Various laboratory data have shown that medicinal plants may possess therapeutic potential in CVDs, and it has been reported that Ginseng, *Ginkgo biloba*, and *Ganoderma lucidum* exert

potential effects against CVDs *in vitro* and *in vivo* [25]. A randomized clinical trial has examined the use of Ginseng, *G. biloba*, and *G. lucidum* to treat CVDs; however, the therapeutic potential of medicinal plants in CVD has not been clinically established. It should be noted that the co-administration of traditional CVD drugs and natural products has shown the potential to suppress CVD, and several clinical trials are ongoing [25]. In our previous study, ginsenosides exhibited potent antiplatelet effects, and their inhibitory concentrations were similar to those of SN [26]. The present has some limitations, given that all experiments were conducted *in vitro*. Although the antiplatelet effect of SN is strong and *C. tricuspidata* affects *ex vivo* platelet aggregation [27], predicting its effect on the human body remains a challenge. Therefore, based on the findings of the present study, we suggest that SN has the potential for clinical application in patients with CVD.

Herein, we found that SN inhibits human platelet aggregation and αIIb/β_3 activation. These results confirm that inside-out signaling molecules inhibit Ca^{2+} mobilization and Ca^{2+} influx. Therefore, SN from *C. tricuspidata* would be useful for patients at a high risk of CVD.

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Conflict of interest The authors declare no conflict of interest.

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