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The inhibitory effects of glabridin on human platelet aggregation and thrombus formation

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Abstract *Glycyrrhiza glabra* is a well-known medicinal herb that grows in various parts of the world and glabridin is a major chemical compound that is found in the root extract of *Glycyrrhiza glabra*. Glabridin is a natural compound known to have antioxidant, anti-inflammatory, anti-atherogenic, anti-osteoporotic and skin-whitening. In this study, we investigated if glabridin inhibited platelet aggregation and thrombus formation. We observed that glabridin inhibited collagen-induced platelet aggregation and suppressed signal transduction molecules such as phosphatidylinositol-3 kinase (PI_3K), Akt, glycogen synthase kinase-3α/β (GSK-3α/β), SYK, cytosolic phospholipase A_2, and p38 expression. In addition, glabridin suppressed thromboxane A_2 generation and thrombin-induced clot retraction. Taken together, glabridin showed strong antiplatelet effects and may be used to block thrombosis- and platelet-mediated cardiovascular diseases.

Keywords Clot retraction · Cyclic nucleotides · Glabridin · Platelet aggregation

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Introduction

Platelets form blood clots to maintain cellular hemostasis. Damaged blood vessels contain exposed collagen fibers which bind to platelet integrin [1]. After binding action, calcium concentration in platelet is increased [2]. Elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) activates Ca^{2+}-dependent kinases which trigger granule release [3]. This agonist-induced signaling cascade activates glycoprotein IIb/IIIa (integrin αIIb/β_3), which then binds to other platelets, and αIIb/β_3-mediated signaling triggers full platelet spreading and aggregation [4]. Platelets are essential cells for hemostasis, but they can also cause thombosis. Hyperactivity of platelets can form a hemostatic plug even with minor stimulation, blocking blood vessels or forming thrombosis. For people exposed to hyperlipidemia, high blood pressure, or cardiovascular disease, this effect can be fatal. Therefore, correct platelet regulation is required to suppress harmful events during cardiovascular disease [5]. However, contrary to expectations, many antiplatelet agents do not ameliorate cardiovascular disease mortality rates [6], which often occur due to sudden symptom onset. Therefore, in an attempt to identify new antithrombosis drugs, we focused on glabridin. Glabridin is a major chemical compound that is found in the root extract of *Glycyrrhiza glabra* and known to have anti-inflammatory, neuroprotection effect, treating bone disease and improving metabolic abnormalities [7]. Regarding cardiovascular disease, studies have reported that glabridin can protect blood vessels by inhibiting LDL oxidation [8]. However, studies have not been reported on the effectiveness of glabridin on the inhibition of platelets and blood clots.

However, the platelet inhibitory effect of glabridin has not been identified. In this study, we evaluated the effects of glabridin using human platelets.

Materials and methods

Materials

Glabridin was purchased from ChemFaces (Wuhan, China). Human platelets were obtained from the Korean Red Cross Blood Center (Suwon, Korea). Fura 2-acetoxymethyl (Fura-2 AM) was purchased from Invitrogen (Eugene, OR, USA). Platelet agonists, collagen, and thrombin were bought from Chrono-Log Co. (Havertown, PA, USA). Phospho-IP_3R, Phospho-VASP (Ser^{157} and Ser^{239}), Phospho-PI_3K, Phospho-Akt (Ser^{473} and Thr^{308}), Phospho-GSK-3α/β, β-actin, phosphor-SYK, phosphor-p38, and phosphor-cytosolic phospholipase A_2 ($cPLA_2$) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Bicinchoninic acid protein assay kit was purchased form Pierce Biotechnology (Rockford, IL, USA). Fibronectin-coated cell adhesion kit as procured from Cell Biolabs (San Diego, CA, USA). A serotonin detection kit was purchased from Labor Diagnostika Nord GmbH and Co. (Nordhorn, Germany). Cyclic nucleotides (cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) assay kits were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Platelet aggregation analyses

Platelets were separated and washed in washing buffer (pH 6.5) and adjusted in suspension buffer (pH 6.9) to 10^8/mL. Glabridin was dissolved in dimethyl sulfoxide (0.1%). Platelets (10^8/mL) were preincubated with different glabridin concentrations (50, 75, 100, and 150 μM) at 37 ºC while stirring, and collagen was added for full platelet aggregation using an aggregometer (Chrono-Log).

Cytotoxicity analyses

We investigated if glabridin concentrations affected lactate dehydrogenase (LDH) levels in platelets. Platelets (10^8/mL) were preincubated with different glabridin concentrations for 15 min at 37 ºC while stirring. After centrifugation at 12,000× *g*, supernatants were separated and LDH levels analyzed using an enzyme-linked immunosorbent assay (ELISA) kit and ELISA plate reader (TECAN, Salzburg, Austria).

Ca^{2+} mobilization analyses

To measure $[Ca^{2+}]_i$, the Grynkiewicz method [9] was used. Platelets were incubated with Fura-2 AM for 20 min, washed, and platelet concentrations adjusted to 10^8/mL using suspension buffer. Platelets (10^8/mL) were incubated with different glabridin concentrations (50, 75, 100, and 150 μM) at 37 ºC for 5 min and then stimulated with collagen (2.5 μg/mL). Ca^{2+} concentrations were analyzed using a fluorescence spectrophotometer (F-2700; Tokyo, Hitachi, Japan).

Serotonin release detection

Platelet aggregation was conducted for 7 min at 37 ºC with glabridin, then reaction cuvette place onto ice in order to terminate release action for 3 min. After termination, the reaction mixture was centrifuged and the supernatant was used. The serotonin was detected using ELISA reader (TECAN).

Analyzing thromboxane A_2 (TXA_2)

Activated platelets synthesize TXA_2 via an "inside-out signaling cascade." TXA_2 acts as a strong agonist and is quickly converted to thromboxane B_2 (TXB_2), which was measured. After collagen-induced platelet aggregation with glabridin, indomethacin was added to stop reactions and mixtures centrifuged briefly to generate TXB_2-containing supernatants, which were analyzed using an ELISA plate reader (TECAN).

Western blotting

To investigate phosphorylation events, platelet aggregation was performed and platelet lysates quantified. Proteins were separated by electrophoresis and then transferred to polyvinylidene fluoride membranes. Primary antibodies were incubated with membranes overnight at 4 ºC, and after washing (Tris-buffered saline plus 0.1% tween 20), a secondary antibody was added and incubated with membranes at room temperature for 2 h. Then, protein signals were developed in a darkroom. Western blotting results were calculated using the Quantity One program (Bio-Rad, Hercules, CA, USA).

Analyzing αIIb/β_3 adhesion to fibronectin

Fibronectin is a plasma protein and functions as an adhesive protein to bind platelet integrin αIIb/β_3. Therefore, we analyzed αIIb/β_3 activity in fibronectin-coated wells. Platelets and different glabridin concentrations (50, 75, 100, and 150 μM) were added to fibronectin-coated wells and stimulated by collagen. In normal reactions, platelets adhere to fibronectin-coated wells to form thin films. After reactions, wells were washed twice in buffer, and platelet layers stained using cell staining solution. After this, extract solution was added to extract stained platelet layers and absorbances analyzed using an ELISA plate reader (TECAN,).to determine platelet adhesion.

Platelet-mediated fibrin clot retraction

Human platelet-rich plasma (300 μL) was incubated with glabridin for 30 min at 37 ºC, and clot retraction was triggered by adding thrombin (0.05 U/mL). After reacting for 15 min, pictures of fibrin clot were taken using a digital camera.

cAMP and cGMP analyses

Platelets (10^8/mL) were preincubated with different glabridin concentrations (50, 75, 100, and 150 μM) for 5 min at 37 ºC. After platelet aggregation was stopped by ethanol (80%), platelets were centrifuged at 500× *g*, and separated supernatants used to determine cyclic nucleotide (cAMP and cGMP) levels using cAMP- and cGMP-ELISA kits and an ELISA plate reader (TECAN).

Data analysis

All data are presented as the mean ± standard deviation with 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 software (SPSS, Chicago, IL, USA) was used for statistical analysis and $p < 0.05$ was considered statistically significant.

Results

Platelet aggregation and cytotoxicity

Glabridin (Fig. 1A) is a natural whitening agent extracted from *Glycyrrhiza Glabra* (Licorice) root. We used collagen (2.5 μg/mL) to promote full platelet aggregation. Human platelets (10^8/mL) were stirred for 2 min with different glabridin concentrations,

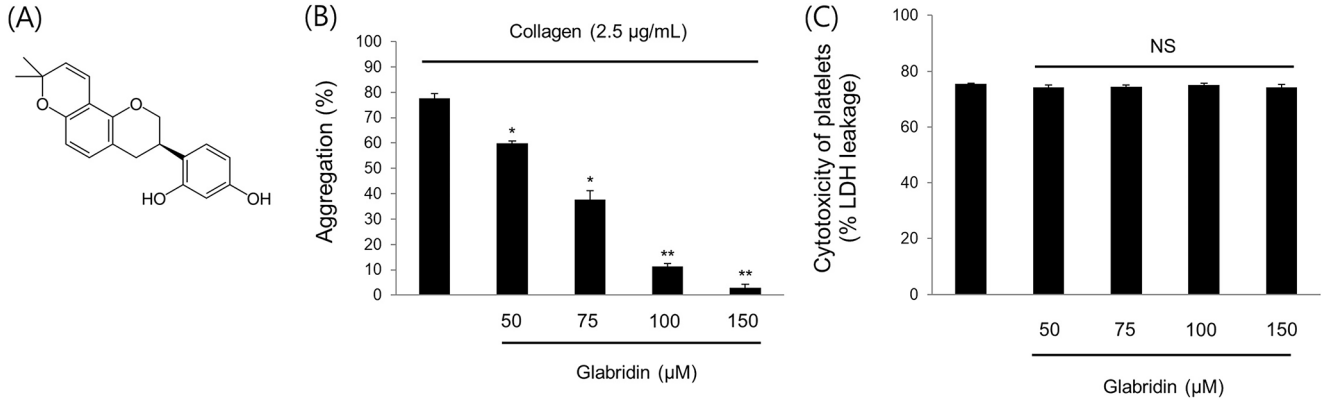

Fig. 1 Glabridin's effects on platelet aggregation. (A) Chemical structure of glabridin (MW. 324.4). (B) Glabridin's effects on collagen-induced human platelet aggregation. (C) Glabridin's effects on cytotoxicity. Data are expressed as the mean ± standard deviation (n =4). *p <0.05, **p <0.01 versus collagen-stimulated human platelets. NS = not significant

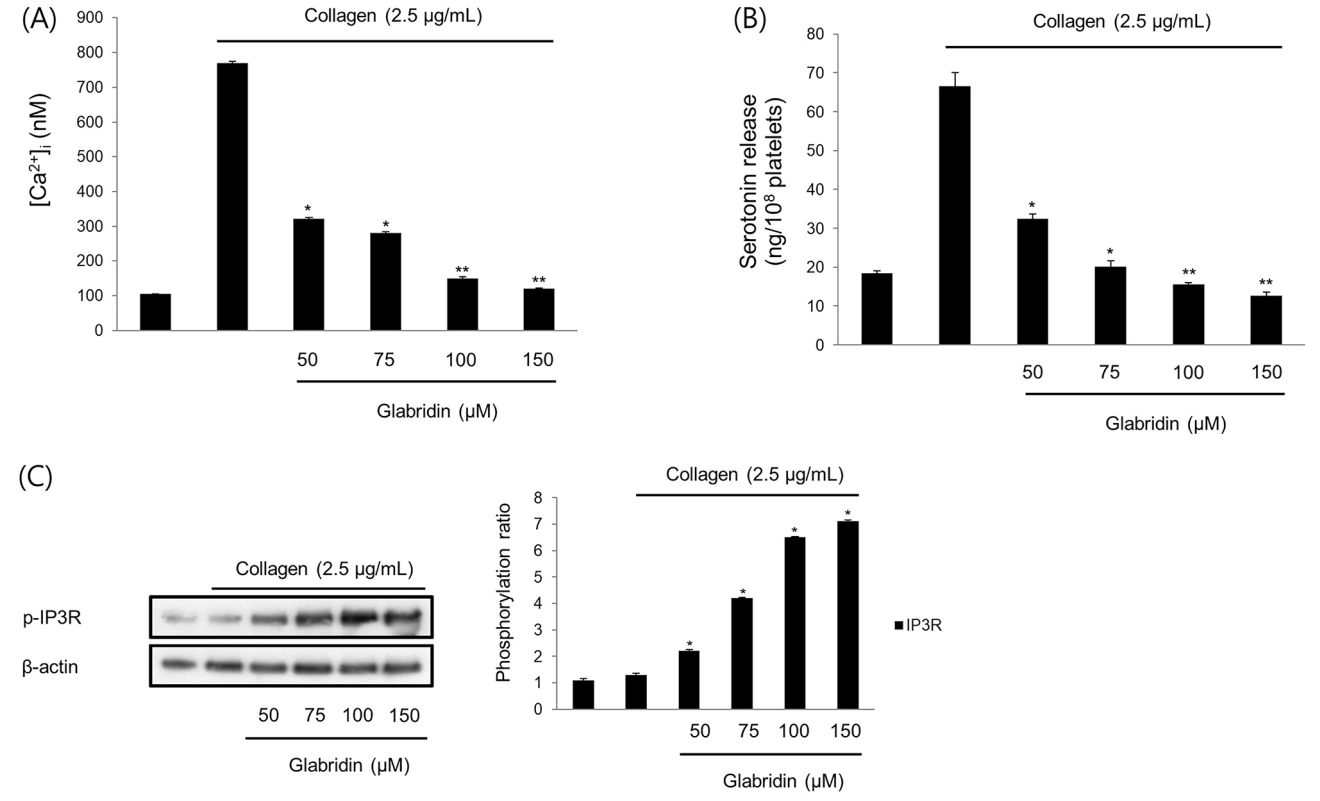

Fig. 2 Glabridin's effects on $[Ca^{2+}]_i$ mobilization, serotonin release and IP_3R phosphorylation. (A) Glabridin's effects on collagen-induced $[Ca^{2+}]_i$ mobilization. (B) Glabridin's effects on collagen-induced serotonin release. (C) Glabridin's effects on collagen-induced IP_3R phosphorylation. Data are expressed as the mean ± standard deviation (n =4). *p <0.05, **p <0.01 versus collagen-stimulated human platelets

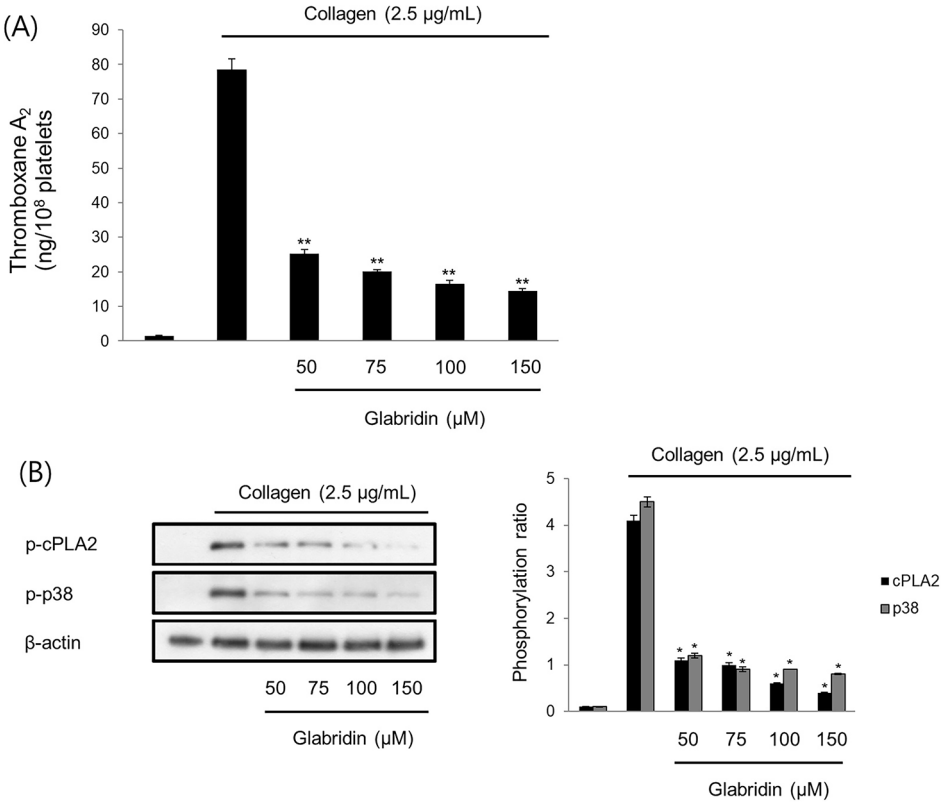

Fig. 3 Glabridin's effects on thromboxane A_2 generation and $cPLA_2$/p38 phosphorylation. (A) Glabridin's effects on collagen-induced thromboxane A_2 generation. (B) Glabridin's effects on collagen-induced $cPLA_2$/p38 phosphorylation. Data are expressed as the mean ± standard deviation (n =4). *p <0.05, **p <0.01 versus collagen-stimulated human platelets

reacted for 5 min, and then collagen added. As shown (Fig. 1B), collagen-induced platelet suspension were strongly aggregated, but were dose-dependently inhibited by glabridin. To confirm cytotoxic effects of glabridin toward platelets, LDH release was analyzed. As shown (Fig. 1C), glabridin did not affect cytotoxicity.

Ca^{2+} mobilization, serotonin secretion and Ca^{2+} receptor phosphorylation

Next, we examined intracellular calcium concentrations and the phosphorylation of Ca^{2+}-related signaling pathway. As shown (Fig. 2A), collagen-increased Ca^{2+} mobilization but was dose-dependently suppressed by glabridin. Increased calcium in platelet activates kinase that triggers granule release, thus we examined whether glabridin affect serotonin and ATP release in δ-granules. As shown in Fig. 2B, glabridin dose-dependently inhibited collagen-stimulated serotonin secretion. Next, we investigated if glabridin could control inositol 1,4,5-trisphosphate receptor (IP_3R) phosphorylation. IP_3R is located on the surface of the endoplasmic reticulum and IP_3R is phosphorylated by cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP)-dependent kinases. We observed that glabridin increased IP_3R phosphorylation when induced by collagen (Fig. 2C).

Thromboxane A_2, $cPLA_2$-, and p38-phosphorylation

TXA_2 acts as an agonist which stimulates platelet activation [10]. As shown (Fig. 3A), TXA_2 was dose-dependently inhibited by glabridin. TXA_2 production is regulated by two signaling molecules, cytosolic phospholipase A_2 ($cPLA_2$) and mitogen-activated protein kinase p38 (p38) [11]. As shown (Fig. 3B), collagen-elevated $cPLA_2$ and p38 phosphorylation was dose-dependently inhibited by glabridin.

αIIb/$β_3$ function and phosphorylation of αIIb/$β_3$-related signaling molecules

Next, we examined αIIb/$β_3$ function, which can affect platelet aggregation and adhesion. αIIb/β3-mediated signaling actually starts as soon as a binding molecules binds to the integrin, allowing various signaling pathways and it makes platelet aggregation more powerful. As shown (Fig. 4A), glabridin suppressed collagen-elevated fibronectin adhesion. Next, we examined the inhibitory effects of glabridin on clot retraction. Fig. 4B shows thrombin-induced fibrin clot formation and contraction. Thrombin induced platelet rich plasma was strongly contracted. However, the retraction was suppressed by glabridin dose-dependently. To confirm glabridin effects on αIIb/$β_3$ activity, we

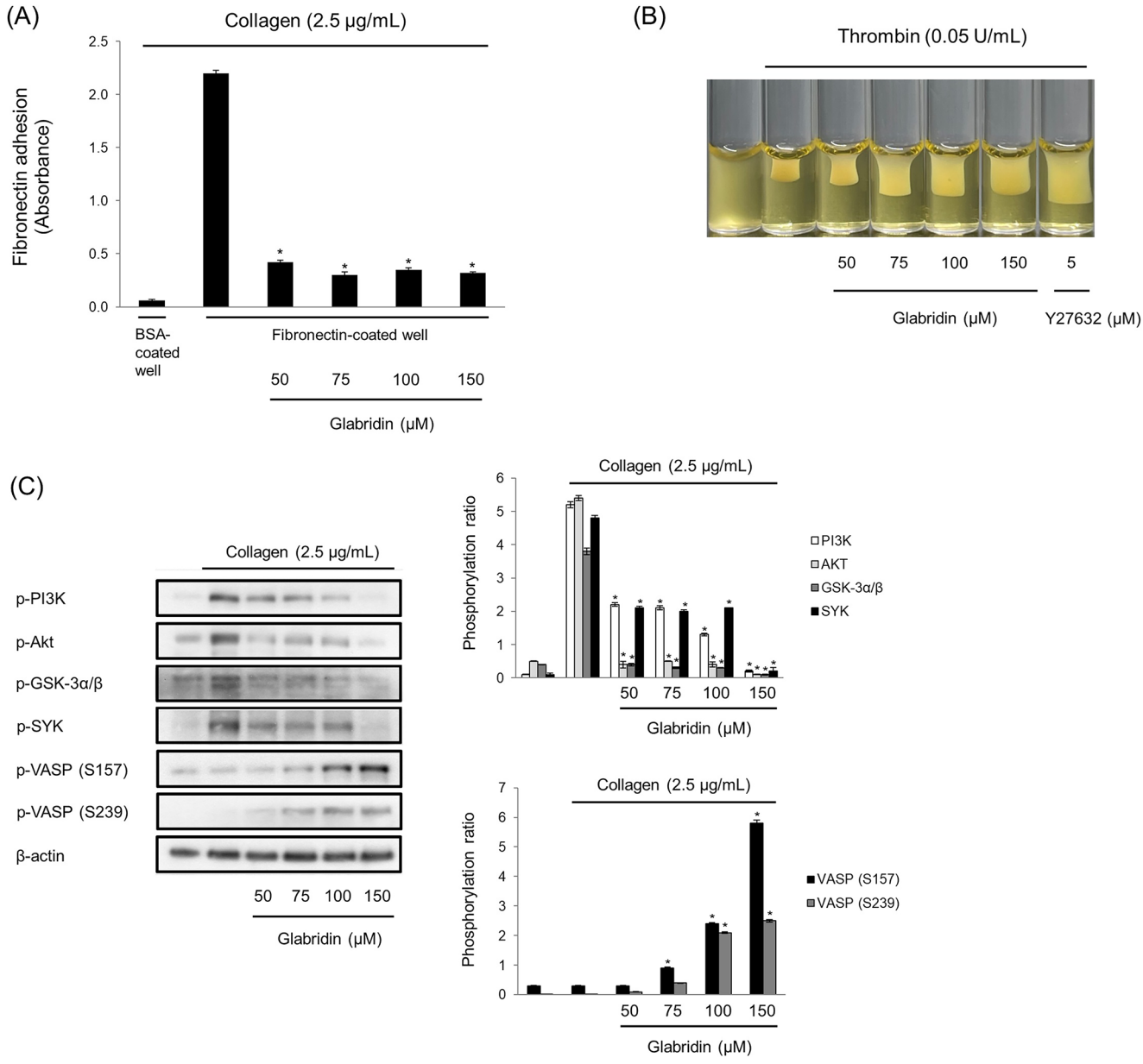

Fig. 4 Glabridin's effects on fibronectin adhesion, clot retraction and PI_3K/Akt/GSK-3α/β, SYK, and VASP phosphorylation. (A) Glabridin's effects on collagen-induced fibronectin adhesion. (B) Glabridin's effects on thrombin-retracted fibrin clot. (C) Glabridin's effects on PI3K/Akt/GSK-3α/β, SYK, and VASP phosphorylation. Data are expressed as the mean ± standard deviation (n =4). *p <0.05, **p <0.01 versus collagen-stimulated human platelets

analyzed the phosphorylation of αIIb/β_3-related signaling molecules (PI_3K/Akt/GSK-3/SYK/VASP), which are essential regulators of the αIIb/β_3-mediated signaling pathways [12,13]. We confirmed that glabridin significantly reduced PI_3K/Akt/GSK-3/SYK-phosphorylation and elevated VASP (Ser^{157}, Ser^{239}) phosphorylation (Fig. 4C).

cAMP and cGMP levels

The most well-known inhibitory molecules secreted form platelets are cyclic nucleotides (cAMP and cGMP), which are synthesized from nitric oxide and prostacyclin in endothelial cells [14]. In platelets, inositol 1, 4, 5-triphosphate receptor (IP_3R), Rap1b, glycoprotein Ibβ, phosphodiesterase 3, and vasodilator-stimulated phosphoprotein (VASP) are major cAMP and cGMP substrates. These signaling molecules can affect $[Ca^{2+}]_i$ mobilization and αIIb/β_3 activity [15]. As shown (Fig. 5A, B), glabridin increased cAMP and cGMP concentrations.

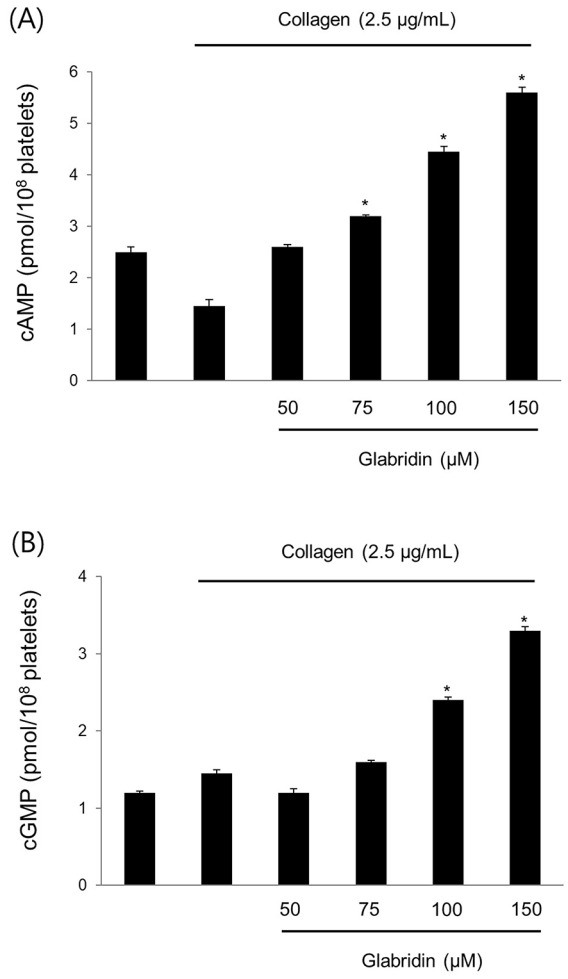

Fig. 5 Glabridin's effects on cAMP and cGMP production. (A) Glabridin's effects on collagen-induced cAMP production. (B) Glabridin's effects on collagen-induced cGMP production. Data are expressed as the mean ± standard deviation (n =4). *p <0.05, **p <0.01 versus collagen-stimulated human platelets

Discussion

In our previous study, *Glycyrrhiza glabra* ethanol extract strongly inhibited collagen-, thrombin, U46619 (TXA_2 analogue)-induced human platelet aggregation. Therefore, we investigated constituent from *Glycyrrhiza glabra* extract and found glabridin, and we used glabridin to identify its antiplatelet activity. In our experiment, glabridin dose-dependently inhibited human platelets stimulated by collagen (Fig. 1B), next, we examined if glabridin regulated $[Ca^{2+}]_i$ mobilization *via* phosphorylation, as shown in Fig. 2A, B, glabridin inhibited $[Ca^{2+}]_i$ mobilization and, serotonin release in δ-granules. We confirmed that glabridin could regulate Ca^{2+} signaling by regulating IP_3R phosphorylation (Fig. 2C) and affect granule release action.

TXA_2 acts as a strong agonist for platelet aggregation, thus we examined if glabridin could regulate TXA_2 production. It is well known that $cPLA_2$ and p38 molecules act in connection with the production of TXA_2. p38 activates $cPLA_2$ via phosphorylation [15] and activated $cPLA_2$ stimulates arachidonic acid release. Next, TXA_2 is created based on arachidonic acid within platelet cytosol [16]. We analyzed the impact of glabridin on the creation of TXA_2 and we confirmed that glabridin suppressed TXA_2 levels *via* the dephosphorylation of $cPLA_2$ and p38 molecules (Fig. 3A, B).

The αIIb/β3-mediated signaling cascade is important in hemostasis. Therefore, we investigated αIIb/β3 activity and αIIb/β3-related signaling molecules by glabridin. As shown in Fig. 4A, glabridin suppressed fibronectin adhesion. Next, we confirmed the integrin inhibitory activity of glabridin through clot retraction test. Thrombin is an agonist that activates platelets and activates at the same time blood coagulation factors to generate fibrin. Platelets aggregate through fibrin-αIIb/β3 interaction and then begin to contract. If glabridin can inhibit the formation of thrombin-induced blood clots, it can be expected to work in actual blood vessels as well. As shown in Fig. 4B, upon thrombin stimulation, platelets contracted together with plasma but glabridin treated PRP showed reduced contraction. PI_3K/Akt/GSK-3 is a signaling molecule that facilitates αIIb/β3 activation [17-20]. SYK is a 72 kDa tyrosine kinase that is stimulated and phosphorylated by platelet agonists and also functions after $αIIb/β_3$ activation and promotes platelet aggregation [21,22]. VASP helps regulate actin filament dynamics and platelet shape, but its phosphorylation inhibits platelet functions. We investigated glabridin effects on PI_3K/Akt/GSK-3 and SYK phosphorylation and showed (Fig. 4C) that glabridin inhibited collagen-elevated PI_3K, Akt (Ser^{473}), GSK-3α/β and SYK phosphorylation. Additionally, glabridin elevated VASP (Ser^{157}, Ser^{239}) phosphorylation. Therefore, glabridin inhibited $αIIb/β_3$ activation *via* $αIIb/β_3$-related signaling molecules.

In normal circulation, the most important second messengers involved in the negative feedback of platelet actions are cAMP and cGMP [23] and are regulated by adenylate, guanylate cyclase, and phosphodiesterases [24,25]. The cAMP/cGMP-dependent protein kinase phosphorylates various substrates in human platelets such as glycoprotein Ibβ subunit, inositol 1,4,5-trisphosphate receptor, Rap1b, caldesmon, and VASP [15]. Therefore, we investigated if glabridin affected cAMP and cGMP production and showed that it increased these levels (Fig. 5A, B).

This study found that glabridin decreased human platelet aggregation, calcium mobilization, fibronectin adhesion, and clot retraction through the regulation of various phosphoproteins and increased cAMP and cGMP levels. Therefore, glabridin would be a useful in antithrombosis applications.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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