Inhibitory effects of isoscooletin on thrombus formation via regulation of cyclic nucleotides in collagen-induced platelets

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Abstract An essential component of the hemostatic process during vascular damage is platelet activation. However, many cardiovascular diseases, such as atherosclerosis, thrombosis, and myocardial infarction, can develop due to excessive platelet activation. Isoscooletin, found primarily in plant roots of the genus *Artemisia* or *Scopolia*, has been studied to demonstrate potential pharmacological effects on Alzheimer's disease and anticancer, but its mechanisms and role in relation to thrombus formation and platelet aggregation have not yet been discovered. This research investigated the effect of isoscooletin on collagen-induced human platelet activation. As a result, isoscooletin strongly increased cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels in a concentration-dependent manner. In addition, isoscooletin greatly phosphorylated inositol 1,4,5-triphosphate receptor (IP₃R) and vasodilator-stimulated phosphoprotein (VASP), known substrates of cAMP-dependent kinase and cGMP dependent kinase. Phosphorylation of IP₃R by isoscooletin induced Ca²⁺ inhibition from the dense tubular system Ca²⁺ channels, and VASP phosphorylation was involved in fibrinogen binding inhibition by inactivating α IIb/ β_3 in the platelet membrane. Isoscooletin finally reduced thrombin-induced fibrin clot production and finally reduced thrombus formation. Therefore, this research suggests that isoscooletin has strong antiplatelet effects and is likely to be helpful for thrombotic diseases involving platelets by acting as a prophylactic and therapeutic agent.

Keywords Cyclic nucleotide · Inositol 1,4,5-triphosphate receptor · Intracellular Ca²⁺ · Isoscooletin · Vasodilator-stimulated phosphoprotein

Introduction

When blood vessels are damaged, the formation of a hemostatic plug depends on the essential response of platelet aggregation. However, excessive interaction between platelets and collagen relating to platelet aggregation can lead to circulatory disorders like atherosclerosis, myocardial infarction, and thrombosis [1]. Collagen supports platelet adhesion to endothelial cells and induces activation of aggregation, secretion and clotting. During collagen-induced platelet activation, an increase in the level of Ca²⁺ ([Ca²⁺]_i) in the cytoplasm of platelets is an important factor causing platelet aggregation. This [Ca²⁺]_i exists in the cell's internal storage, called a dense tubular system. It is known that calcium ions are mobilized into the cytoplasm when inositol 1,4,5-triphosphate (IP₃) binds to a receptor located in the cell's internal storage [2]. The increased [Ca²⁺]_i phosphorylates the myosin light chain (20 kDa) that relies on the Ca²⁺/calmodulin complex and activates protein kinase C (PKC) in cooperation with diacylglycerol (DG). As a result, phosphorylation of pleckstrin occurs, leading to the rearrangement of cytoskeletal proteins and finally platelet aggregation [2].

In addition, DG produced by the degradation of one of the platelet membrane molecules, phosphatidylinositol 4,5-bisphosphate (PIP₂), is continuously hydrolyzed by DG lipase and monoacylglycerol lipase to produce arachidonic acid and converted to thromboxane A₂ (TXA₂) [3]. TXA₂ is known to induce platelet secretion and morphological changes by activating platelets [4]. As a matter of fact, the TXA₂ analogue, U46619 (9,11-dideoxy-9a,11a-methanoeperoxy prostaglandin F_{2a}), has proved to be an effective platelet aggregation inducer that increases [Ca²⁺]_i resulting in the phosphorylation of pleckstrin and myosin light chain [5,6].

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This is the normal process of haemostatic reaction of blood, but if excessive platelet aggregation occurs, various vascular diseases, such as atherosclerosis, can develop. Therefore, appropriate inhibition of platelet activation may be a useful approach to the prevention of cardiovascular disease [5,6].

Antiplatelet agents, verapamil and theophylline, are known to function by increasing the level of circulating adenosine monophosphate (cAMP), which reduces $[Ca^{2+}]_i$, an essential component of platelet aggregation [7]. In addition, platelet cyclic guanosine monophosphate (cGMP) levels are increased using cGMP phosphodiesterase (PDE) inhibitors such as zaprinast and erythro-9-(2-hydroxy-3-nonyl) adenine, and vasodilators such as nitroprusside and molsidomine [8]. During normal blood circulation, vascular endothelial cells let out prostaglandin I_2 and nitric oxide leading to cAMP and cGMP production from platelets. Increased cAMP level induces activation of protein kinase A (PKA), while an increase in cGMP level induces activation of protein kinase G (PKG). PKA and PKG are known to cause phosphorylation of the substrate proteins vasodilator-stimulated phosphoprotein (VASP) and inositol 1,4,5-triphosphate receptor (IP_3R) [9]. Phosphorylation of IP_3R inactivates the receptor, inhibiting dense tubular system Ca^{2+} mobilization into the cytoplasm [7,10]. In platelets, VASP is the main substrate of PKA and PKG, and assists in modulating the activation of $\alpha IIb/\beta_3$ and actin filament activity. Phosphorylation of cGMP-dependent VASP Ser²³⁹ or phosphorylation of cAMP-dependent VASP Ser¹⁵⁷ can lead to inhibition of $\alpha IIb/\beta_3$ activation as well as actin filament elongation inhibition [11,12]. Consequently, the phosphorylation of IP_3R is essential to confirm the antiplatelet effect since it inhibits Ca^{2+} mobilization and the phosphorylation of VASP is essential to confirm the antiplatelet effect since it inhibits the activity of platelets through $\alpha IIb/\beta_3$ inhibition.

Isoscopoletin, found primarily in plant roots of the genus *Artemisia* or *Scopolia*, has been studied for medicinal properties that may prove beneficial for Alzheimer's disease and anti-cancer [13,14]. However, the role and mechanism of 6-hydroxy-7-methoxy-2H-chromen-2-one (Isoscopoletin) in human platelet activation is unknown. To elucidate the antiplatelet effect of isoscopoletin, we examined the actions of isoscopoletin on calcium mobilization through the regulation of cyclic nucleotides, and thrombus formation through fibrinogen binding. We also investigated whether isoscopoletin is involved ultimately in platelet activation and thrombus formation. If isoscopoletin is shown to be an effective antiplatelet agent, it is expected that isoscopoletin will contribute to the CVD prevention caused by thrombosis.

Materials and Methods

Materials

Isoscopoletin was provided from Avention Corporation (Seoul, Korea) (Fig. 1). Isoscopoletin was dissolved in dimethyl sulfoxide (DMSO) solution to 0.25% finally, and the same concentration of

Fig. 1 The structure of isoscopoletin. PIN: 6-hydroxy-7-methoxy-2H-chromen-2-one, Chemical formula: $C_{10}H_8O_4$, Molar mass: 192.17 g/mol

DMSO was added to the control not pretreated with isoscopoletin. The Enzyme Immunoassay (EIA) kits for cAMP and cGMP were provided by Cayman Chemical (Ann Arbor, MI, USA). Collagen was bought from Chrono-Log Corporation (Havertown, PA, USA). Fura 2-AM was bought from Invitrogen (Eugene, OR, USA). Anti-phospho- IP_3R type I, Anti-VASP, Anti-phospho-VASP Ser157, Anti-phospho-VASP Ser239, anti- β -actin, anti-rabbit IgG-HRP-conjugate, and lysis buffer were provided by Cell Signaling (Beverly, MD, USA). Polyvinylidene difluoride (PVDF) membranes was provided by Thermo fisher scientific Corporation (Middlesex County, MA, USA). Invitrogen Molecular Probes supplied Enhanced chemiluminescence solution (ECL) and Fibrinogen Alexa Fluor 488 conjugate.

Preparation of human washed platelets

Korean Red Cross Blood Source (Suwon, Korea) supplied Human platelet-rich plasma (PRP). The human washed platelets were prepared according to the previously performed method [15]. To obtain the concentrate platelets, PRP was centrifuged for 10 minutes at $1,300 \times g$ and washed with buffer (12 mM $NaHCO_3$, 2.7 mM KCl, 0.36 mM NaH_2PO_4 , 138 mM NaCl, 1 mM Na_2EDTA , 5. mM glucose, and pH 6.9). Washed twice, the platelets suspended with suspension buffer (12 mM $NaHCO_3$, 2.7 mM KCl, 0.36 mM NaH_2PO_4 , 138 mM NaCl, 5.5 mM glucose, 0.49 mM $MgCl_2$, 0.25% gelatin, and pH 7.4) to a final concentration of 10^8 cells/mL. The above procedure was performed at room temperature to experiment while avoiding platelet aggregation at low temperature, and approval was obtained from the Institutional Review Board (IRB) of the University of Namseoul to perform this experiment (1041479-HR-201803-003).

Measurement of cyclic nucleotides (cAMP and cGMP) production

Washed platelets (10^8 cells/mL) incubation occurred for 3 minutes at $37^\circ C$, 2 mM $CaCl_2$ was added, followed with stimulation by collagen (2.5 $\mu g/mL$) to induce aggregation for 5 minutes. Reaction termination happened with the addition of 1 M HCl, and cAMP or cGMP was measured using an EIA kit through a Synergy HT Multi-Model Microplate Reader (BioTek Instruments, Winooski, Vermont, USA).

Measurement of intracellular Ca^{2+} mobilization

5 μM of Fura 2-AM was incubated with the PRP at $37^\circ C$ for 60 minutes, prepared from washed platelets (10^8 cells/mL) following

the process mentioned above, and 2 mM CaCl_2 was added to incubate at 37 °C for 3 min, stimulated with collagen (2.5 $\mu\text{g/mL}$), and measured for 5 min. Measuring of Fura 2 fluorescence was done with a spectral fluorescence photometer (SFM 25, Winooski, VT, USA) of BioTec Instrument (SFM 25). A 340 nm initial excitation wavelength was set. With the ultimate excitation wavelength reaching 510 nm, increasing in increments of 0.5 seconds. The emission wavelength used a 510 nm setting. The amount of Ca^{2+} mobilization was calculated with Grynkiewicz's method [16].

Immunoblotting

The reaction was terminated by adding 1x lysis buffer. The protein concentration of platelet lysates were measured using a BCA protein kit (Pierce Biotechnology, IL, USA). Protein (20 μg) was separated via 4–20% SDS-PAGE and transferred to PVDF membrane. A dilution factor of 1:1,000 was used to treat the primary antibody, and a dilution factor of 1:2,000 was used to treat the secondary antibody. Visualization was done using ECL reagent (Invitrogen Molecular Probes).

Fibrinogen binding measurement

After treatment of washed platelets (10^8 cells/mL) with 2 mM CaCl_2 and Fibrinogen Alexa Fluor 488 conjugate (30 $\mu\text{g/mL}$), stimulation with collagen (2.5 $\mu\text{g/mL}$) was measured for 5 minutes. The reaction termination was carried out with the addition of phosphate-buffered saline (PBS, pH 7.4) with 0.5% paraformaldehyde. The above process was performed in state of blocking light, and the binding of fibrinogen was measured with FACS and analyzed with Cell-Quest software (BD Bioscience, San Jose, CA, USA).

Platelet mediated fibrin clot formation measurement

Transferring of PRP (500 μL) to a polyethylene tube was done to prevent adhesion and stimulated for 15 min at 37 °C with thrombin (0.05 U/mL) and the addition of 2 mM CaCl_2 . Pictures of the fibrin clot were collected with a digital camera. Calculation of the coagulation area was completed using ImageJ Software (v1.46, National Institutes of Health, Bethesda, MD, USA).

Statistical analyses

Experimental results are presented as mean $\pm$ standard deviation. The Student's t-test or ANOVA was used for statistical analysis, and if $p < 0.05$, the value was considered statistically significant. If there was a significant difference between the group means according to the ANOVA, the each group was compared using Scheffé's method.

Results

Effects of isoscapolein on the production of cyclic nucleotides

The effect of isoscapolein on cAMP or cGMP production was confirmed. As shown in Fig. 2A, isoscapolein heightened cAMP

Fig. 2 Effects of isoscapolein on cyclic nucleotides production. (A) Effects of isoscapolein on cAMP production stimulated by collagen. (B) Effects of isoscapolein on cGMP production stimulated by collagen. Data are expressed as means $\pm$ SD ($n = 4$). * $p < 0.05$, ** $p < 0.001$ compared with the collagen-stimulated platelets

production from 3.92 ± 0.41 pmol/ 10^8 platelets to 8.74 ± 0.74 pmol/ 10^8 platelets, and cGMP production from 6.53 ± 0.46 pmol/ 10^8 platelets to 17.09 ± 1.98 pmol/ 10^8 platelets significantly (Fig. 2B). In particular, with a 100 μM isoscapolein concentration or higher, the significance was clearly observed, and a stronger increase in cGMP than cAMP was observed. These results show that in collagen-induced platelets, isoscapolein inhibits the platelet activation, significantly increasing the production of cAMP and cGMP.

Effects of isoscapolein on intracellular Ca^{2+} mobilization and IP_3R phosphorylation

Knowing that intracellular Ca^{2+} mobilization ($[\text{Ca}^{2+}]_i$) is crucial for the platelet activation, this study verified the effects of isoscapolein on $[\text{Ca}^{2+}]_i$. The level of $[\text{Ca}^{2+}]_i$ was increased from 101.2 ± 0.8 to 632.1 ± 16.8 nM by collagen (Fig. 3A). However, isoscapolein (100–500 μM) significantly decreased the concentration of $[\text{Ca}^{2+}]_i$ increased by collagen in a concentration-dependently. The inhibition rate of $[\text{Ca}^{2+}]_i$ of isoscapolein (500 μM) was up to 82.3%. Furthermore, this study verified the effect isoscapolein had on phosphorylation of $[\text{Ca}^{2+}]_i$ - regulating protein IP_3R . As

Fig. 3 Effects of isoscapolein on [Ca²⁺]_i mobilization and IP₃R phosphorylation. (A) Inhibitory effects of isoscapolein on collagen-induced [Ca²⁺]_i mobilization. (B) Effects of isoscapolein on collagen-induced IP₃R phosphorylation. Measurement of [Ca²⁺]_i mobilization and Western blotting was described in “Materials and Methods” section. The data are expressed as the mean ± standard deviation (n=4). ^a*p* < 0.05 compared with no-stimulated platelets, ^{*}*p* < 0.05, ^{**}*p* < 0.001 compared with the collagen-stimulated platelets

shown in Fig. 3B, isoscapolein (100–500 µM) increased, concentration-dependently, IP₃R phosphorylation in collagen-induced platelets, and it was confirmed that there were significant results at concentrations above 100 µM. These results indicate that the decrease in intracellular Ca²⁺ mobilization by isoscapolein is due to IP₃R phosphorylation.

Effects of isoscapolein on VASP Phosphorylation

Because isoscapolein increased production of both cAMP and cGMP in collagen-induced platelets in a concentration-dependently (Fig. 2), isoscapolein in this study might affect the phosphorylation of both cAMP and cGMP dependent VASP Ser¹⁵⁷ and Ser²³⁹ in

Fig. 4 Effects of isoscapolein on VASP phosphorylation. Western blotting was determined as described in “Materials and Methods” section. The data are expressed as the mean ± standard deviation (n=4). ^a*p* < 0.05 compared with no-stimulated platelets, ^{*}*p* < 0.05, ^{**}*p* < 0.001 compared with the collagen-stimulated platelets

collagen-induced platelets. As shown in Fig. 4, VASP Ser¹⁵⁷ and VASP Ser²³⁹ phosphorylation were increased significantly with increasing concentration of isoscapolein. Specifically, it was established that the significance was clearly observed at a concentration of 100 µM or more, and the extent of phosphorylation of VASP Ser²³⁹ was stronger than that of VASP Ser¹⁵⁷. This result is consistent with the trend of heightened cAMP and cGMP production by isoscapolein, showing that the increase in cAMP and cGMP production led to phosphorylation of VASP.

Effects of isoscapolein on fibrinogen binding to αIIb/β₃

Because isoscapolein heightened phosphorylation of VASP Ser¹⁵⁷ and VASP Ser²³⁹ by means of increased cAMP and cGMP, the effects of isoscapolein on the fibrinogen binding capacity to αIIb/β₃ was confirmed in this study. Collagen increased fibrinogen binding to αIIb/β₃ at a rate of 84.8 ± 1.1% (Fig. 5A-b, 5B) than intact cell (0.6 ± 0.2%). However, it was confirmed that the fibrinogen binding was inhibited by isoscapolein in a concentration-dependently, and in particular, the significance was clearly shown at a concentration of 100 µM or higher. In addition, the inhibition rate of isoscapolein (500 µM) was confirmed to be 80.2% (Fig. 5A-c-e, 5B).

Effects of isoscapolein on platelet-mediated fibrin clot formation

Over time, as the binding of fibrinogen is increased by activation of αIIb/β₃ through a platelet activator, fibrin clot is formed through an external αIIb/β₃ signal transduction pathway. Therefore,

Fig. 5 Effects of isoscooletin on collagen-induced fibrinogen binding. (A) The flow cytometry histograms on fibrinogen binding. a, Intact platelets (base); b, collagen (2.5 µg/mL); c, collagen (2.5 µg/mL) + isoscooletin (100 µM); d, collagen (2.5 µg/mL) + isoscooletin (300 µM); e, collagen (2.5 µg/mL) + isoscooletin (500 µM). (B) Effects of isoscooletin on collagen-induced fibrinogen binding (%). Measurement of fibrinogen binding was described in “Materials and Methods” section. The data are expressed as the mean ± standard deviation (n=4). ^a*p* < 0.05 compared with non-stimulated platelets, **p* < 0.05, ***p* < 0.001 compared with the collagen-stimulated platelets

this study measured the effects of isoscooletin on thrombin-activated fibrin clot formation. As shown in Fig. 6A, fibrin clot was greatly formed by thrombin, and isoscooletin (100, 300 and 500 µM) inhibited thrombin-induced formation of fibrin clot in a concentration-dependent manner, especially in concentrations above 100 µM. The inhibition rates of isoscooletin (100, 300 and 500 µM) were confirmed to be 22.2, 57.4, and 77.5%, respectively (Fig. 6B).

Discussion

When platelet activation occurs, the activated phospholipase C- γ_2 (PLC- γ_2) hydrolyze Phosphatidylinositol 4,5-bisphosphate (PIP₂) on the platelet membrane. This results in diacylglycerol (DG) as

well as inositol 1,4,5-triphosphate (IP₃). The produced IP₃ let go Ca²⁺ mobilization into cells from dense tubular system ([Ca²⁺]_i) and relies on DG-dependent PKC for activation [17]. Platelet activation results from phosphorylated Ca²⁺/calmodulin-dependent proteins, pleckstrin (40 or 47 kDa) and myosin light chain (20 kDa), which is caused by the increased [Ca²⁺]_i [18].

On the other hand, cyclic nucleotides (cAMP and cGMP) are known to suppress platelet aggregation through a reduction in [Ca²⁺]_i, and cause activation of cAMP-dependent protein kinase (PKA) in addition to cGMP-dependent protein kinase (PKG) [19]. In this study, isoscooletin significantly heightened cAMP and cGMP production and inhibited [Ca²⁺]_i in platelets concentration-dependently. These results indicate that cyclic nucleotides, with increased production by isoscooletin, play a central role in the inhibition of platelet activity through the reduction of [Ca²⁺]_i. The

Fig. 6 Effects of isoscoipoletin on fibrin clot retraction. (A) Effects of isoscoipoletin on thrombin-retracted fibrin clot photographs (B) Effects of isoscoipoletin on thrombin-retracted fibrin clot area. Quantification of fibrin clot retraction was described in “Materials and Methods” section. The data are expressed as the mean $\pm$ standard deviation ($n=4$). ^a $p < 0.05$ compared with no-stimulated platelets, $*p < 0.05$, $**p < 0.001$ compared with the thrombin-stimulated platelets

heightened cAMP and cGMP are used to phosphorylate several substrate proteins by activating PKA and PKG, and are known to make an effect on IP₃ receptor (IP₃R) through phosphorylation of substrates [9].

As shown in Fig. 3A, isoscoipoletin strongly phosphorylates IP₃R in a concentration-dependently. The result demonstrates that the activation of PKA and PKG resulting from isoscoipoletin led to the phosphorylation of IP₃R, thus preventing the Ca²⁺ channel in dense tubular system from opening and reducing [Ca²⁺]_i. In addition, the increased cAMP and cGMP due to isoscoipoletin led to phosphorylation of their substrate, VASP, via the activation of PKA and PKG. It is known that VASP functions as a vital substrate of cAMP/cGMP-dependent PKA/PKG which regulates the activation of platelets through the control of platelet adhesion and secretion properties, and the phosphorylated VASP inhibits integrin α IIb/ β ₃ activation causing the inhibition of platelet aggregation [20,21].

In this research, isoscoipoletin significantly inhibited fibrinogen binding to α IIb/ β ₃ on collagen-induced platelets. The phosphorylation of PKA-dependent VASP Ser¹⁵⁷ and PKG-dependent VASP Ser²³⁹ by activation of PKA and PKG is thought to be due to

isoscoipoletin, which could raise the levels of cAMP and cGMP. Furthermore, integrin α IIb/ β ₃-mediated signal transduction generally causes platelet cytoskeleton deformation, which makes effects on platelet proliferation and thrombus production. Thrombus production is the most important point during the repair on damaged parts of blood vessels, and activated platelets are accumulated in damaged blood vessels resulting in fibrin platelet meshwork development. The formation of fibrin clot acting on hemostasis of damaged blood vessels initiates contraction for a duration of 30 to 60 minutes and results in the creation of a thrombus by pulling the created plug. An important role in fibrin clot formation is the interaction between α IIb/ β ₃ and fibrinogen, and α IIb/ β ₃ activity inhibitors have been shown to greatly inhibit thrombus formation [22]. Thrombin activates platelets integrin to increase the binding capacity of fibrinogen to α IIb/ β ₃, resulting in clot production. As shown in Fig. 6, the antiplatelet effects of isoscoipoletin caused the suppression of thrombin-activated fibrin clot, which is the underlying reason for thrombosis inhibition, in a concentration-dependently. This result shows that isoscoipoletin is likely to be a potent antiplatelet agent that heighten cAMP and cGMP production, and thus inhibit thrombus production.

In next studies, research is needed on the mechanism by which isoscoipoletin heightened cAMP and cGMP production. According to a previous study, scoparone, a substance with a structure similar to isoscoipoletin, has been reported to promote penile erection through vasodilation due to an increase in nitric oxide (NO) and an increase in cGMP production [23]. NO is known to increase the production of cGMP by activating NO sensitive guanylyl cyclase (NO-GC) in platelets. This effect could cause cGMP production with the increase of activated NO-GC, promoting thrombolysis [24]. In line with the results of this experiment, it was confirmed that isoscoipoletin significantly increased cGMP production, possibly resulting from an increase in NO by isoscoipoletin. In addition, cyclic nucleotides phosphodiesterase (PDE) or adenylyl cyclase/guanylyl cyclase activation is a factor for cAMP and cGMP levels [25]. Since the PDE activity inhibition increases cyclic nucleotides levels during platelet aggregation, therapeutic effects on thrombosis have been reported with the use of PDE inhibitors [26]. As expected, triple rasal, cilostazol, and dipyridamole, all of which act as PDE inhibitors, have been used as the antiplatelet agents that clinically increase the level of cyclic nucleotides [8]. Isoscoipoletin is likely to be developed as a drug that acts as an antiplatelet through a similar mechanism.

In conclusion, isoscoipoletin induces IP₃R and VASP phosphorylation in human platelets by heightening cAMP and cGMP, significantly inhibiting both the mobilization of Ca²⁺ into the cytoplasm and activation of integrin α IIb/ β ₃, and finally, thrombin-induced fibrin clot production was inhibited concentration-dependently. Therefore, this research suggests that isoscoipoletin can be effectively developed as antithrombotic and therapeutic agents.

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Conflict of interest The authors of this paper have declared no conflicts of interest.

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