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

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Received: 23 July 2020 / Accepted: 24 August 2020 / Published Online: 30 September 2020
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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. Isoscopoletin, 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 isoscopoletin on collagen-induced human platelet activation. As a result, isoscopoletin strongly increased cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels in a concentration-dependent manner. In addition, isoscopoletin greatly phosphorylated inositol 1,4,5-triphosphate receptor (IP$_3$R) and vasodilator-stimulated phosphoprotein (VASP), known substrates of cAMP-dependent kinase and cGMP dependent kinase. Phosphorylation of IP$_3$R by isoscopoletin induced Ca$^{2+}$ inhibition from the dense tubular system Ca$^{2+}$ channels, and VASP phosphorylation was involved in fibrinogen binding inhibition by inactivating $\alpha_{IIb}\beta_3$ in the platelet membrane. Isoscopoletin finally reduced thrombus formation. Therefore, this research suggests that isoscopoletin 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 Ca$^{2+}$ · Isoscopoletin · 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 Ca$^{2+}$ ([Ca$^{2+}$]$_i$) in the cytoplasm of platelets is an important factor causing platelet aggregation. This [Ca$^{2+}$]$_i$ 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 (IP$_3$) binds to a receptor located in the cell's internal storage [2]. The increased [Ca$^{2+}$]$_i$ phosphorylates the myosin light chain (20 kDa) that relies on the Ca$^{2+}$/calmodulin complex and activates protein kinase C (PKC) in cooperation with diacy glycerol (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 (PIP$_2$), is continuously hydrolyzed by DG lipase and mono acylglycerol lipase to produce arachidonic acid and converted to thromboxane A$_2$ (TXA$_2$) [3]. TXA$_2$ is known to induce platelet secretion and morphological changes by activating platelets [4]. As a matter of fact, the TXA$_2$ analogue, U46619 (9,11-dideoxy-9a.1la-methanoepoxy prostaglandin F2a), has proved to be an effective platelet aggregation inducer that increases [Ca$^{2+}$]$_i$, resulting in the phosphorylation of pleckstrin and myosin light chain [5,6].
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\(^{2+}\)], 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 erythromycin 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\(_{3}\))R [9]. Phosphorylation of IP\(_{3}\)R 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 αIIbβ\(_3\) and actin filament activity. Phosphorylation of cGMP-dependent VASP Ser\(^{197}\) or phosphorylation of cAMP-dependent VASP Ser\(^{157}\) can lead to inhibition of αIIbβ\(_3\) activation as well as actin filament elongation inhibition [11,12]. Consequently, the phosphorylation of IP\(_{3}\)R 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 αIIbβ\(_3\) inhibition.

Isoscopoletin, found primarily in plant roots of the genus Artemisia or Scopola, 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 (Isocopoletin) 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 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\(_{3}\)R type 1, Anti-VASP, Anti-phospho-VASP Ser\(^{157}\), Anti-phospho-VASP Ser\(^{239}\), 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 Thermos 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× g and washed with buffer (12 mM NaHCO\(_3\), 2.7 mM KCl, 0.36 mM Na\(_2\)HPO\(_4\), 138 mM NaCl, 1 mM Na\(_2\)EDTA, 5 mM glucose, and pH 6.9). Washed twice, the platelets were suspended with suspension buffer (12 mM NaHCO\(_3\), 2.7 mM KCl, 0.36 mM Na\(_2\)HPO\(_4\), 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 °C, 2 mM CaCl\(_2\) was added, followed with stimulation by collagen (2.5 μg/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 °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 μg/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 μg/mL), stimulation with collagen (2.5 μg/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 ± 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 Scheffe’s method.

**Results**

**Effects of isoscopoletin on the production of cyclic nucleotides**

The effect of isoscopoletin on cAMP or cGMP production was confirmed. As shown in Fig. 2A, isoscopoletin heightened cAMP 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 isoscopoletin 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, isoscopoletin inhibits the platelet activation, significantly increasing the production of cAMP and cGMP.

**Effects of isoscopoletin on intracellular Ca$^{2+}$ mobilization and IP$_3$R phosphorylation**

Knowing that intracellular Ca$^{2+}$ mobilization ([Ca$^{2+}$]) is crucial for the platelet activation, this study verified the effects of isoscopoletin on [Ca$^{2+}$]. The level of [Ca$^{2+}$], was increased from 101.2±0.8 to 362.1±16.8 nM by collagen (Fig. 3A). However, isoscopoletin (100–500 μM) significantly decreased the concentration of [Ca$^{2+}$] increased by collagen in a concentration-dependently. The inhibition rate of [Ca$^{2+}$], of isoscopoletin (500 μM) was up to 82.3%. Furthermore, this study verified the effect isoscopoletin had on phosphorylation of [Ca$^{2+}$] - regulating protein IP$_3$R. As
shown in Fig. 3B, isoscopoletin (100-500 μM) increased, concentration-dependently, IP<sub>R</sub> 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 Ca<sup>2+</sup> mobilization by isoscopoletin is due to IP<sub>R</sub> phosphorylation.

**Effects of isoscopoletin on VASP Phosphorylation**

Because isoscopoletin increased production of both cAMP and cGMP in collagen-induced platelets in a concentration-dependently (Fig. 2), isoscopoletin in this study might affect the phosphorylation of both cAMP and cGMP dependent VASP Ser<sup>157</sup> and Ser<sup>239</sup> in collagen-induced platelets. As shown in Fig. 4, VASP Ser<sup>157</sup> and VASP Ser<sup>239</sup> phosphorylation were increased significantly with increasing concentration of isoscopoletin. 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 Ser<sup>239</sup> was stronger than that of VASP Ser<sup>157</sup>. This result is consistent with the trend of heightened cAMP and cGMP production by isoscopoletin, showing that the increase in cAMP and cGMP production led to phosphorylation of VASP.

**Effects of isoscopoletin on fibrinogen binding to αIIb/β<sub>3</sub>**

Because isoscopoletin heightened phosphorylation of VASP Ser<sup>157</sup> and VASP Ser<sup>239</sup> by means of increased cAMP and cGMP, the effects of isoscopoletin on the fibrinogen binding capacity to αIIb/β<sub>3</sub> was confirmed in this study. Collagen increased fibrinogen binding to αIIb/β<sub>3</sub> 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 isoscopoletin 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 isoscopoletin (500 μM) was confirmed to be 80.2% (Fig. 5A-c-e, 5B).

**Effects of isoscopoletin on platelet-mediated fibrin clot formation**

Over time, as the binding of fibrinogen is increased by activation of αIIb/β<sub>3</sub> through a platelet activator, fibrin clot is formed through an external αIIb/β<sub>3</sub> signal transduction pathway. Therefore,
this study measured the effects of isoscopoletin on thrombin-activated fibrin clot formation. As shown in Fig. 6A, fibrin clot was greatly formed by thrombin, and isoscopoletin (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 isoscopoletin (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-γ2 (PLC-γ2) 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, isoscopoletin significantly heightened cAMP and cGMP production and inhibited [Ca2+]i, in platelets concentration-dependently. These results indicate that cyclic nucleotides, with increased production by isoscopoletin, play a central role in the inhibition of platelet activity through the reduction of [Ca2+]i. The
heightened cAMP and cGMP are used to phosphorylate several substrate proteins by activating PKA and PKG, and are known to make an effect on IP$_3$ receptor (IP$_3$R) through phosphorylation of substrates [9].

As shown in Fig. 3A, isoscopoletin strongly phosphorylates IP$_3$R in a concentration-dependently. The result demonstrates that the activation of PKA and PKG resulting from isoscopoletin led to the phosphorylation of IP$_3$R, thus preventing the Ca$^{2+}$ channel in dense tubular system from opening and reducing [Ca$^{2+}$]. In addition, the increased cAMP and cGMP due to isoscopoletin 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 αIIbβ3 activation causing the inhibition of platelet aggregation [20,21].

In this research, isoscopoletin significantly inhibited fibrinogen binding to αIIbβ3 on collagen-induced platelets. The phosphorylation of PKA-dependent VASP Ser$^{157}$ and PKG-dependent VASP Ser$^{239}$ by activation of PKA and PKG is thought to be due to isoscopoletin, which could raise the levels of cAMP and cGMP. Furthermore, integrin αIIbβ3-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 αIIbβ3 and fibrinogen, and αIIbβ3 activity inhibitors have been shown to greatly inhibit thrombus formation [22]. Thrombin activates platelets integrin to increase the binding capacity of fibrinogen to αIIbβ3, resulting in clot production. As shown in Fig. 6, the antiplatelet effects of isoscopoletin caused the suppression of thrombin-activated fibrin clot, which is the underlying reason for thrombosis inhibition, in a concentration-dependently. This result shows that isoscopoletin 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 isoscopoletin heightened cAMP and cGMP production. According to a previous study, scoparone, a substance with a structure similar to isoscopoletin, 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 isoscopoletin significantly increased cGMP production, possibly resulting from an increase in NO by isoscopoletin. In addition, cyclic nucleotides phosphodiesterase (PDE) or adenylyl cyclase/guanyl 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 rusal, cilostazol, and dipyridamole, all of which act as PDE inhibitors, have been used as the antplatelet agents that clinically increase the level of cyclic nucleotides [8]. Isoscopoletin is likely to be developed as a drug that acts as an antplatelet through a similar mechanism.

In conclusion, isoscopoletin induces IP$_3$R and VASP phosphorylation in human platelets by heightening cAMP and cGMP, significantly inhibiting both the mobilization of Ca$^{2+}$ into the cytoplasm and activation of integrin αIIbβ3, and finally, thrombin-induced fibrin clot production was inhibited concentration-dependently. Therefore, this research suggests that isoscopoletin can be effectively developed as antithrombotic and therapeutic agents.
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