Inhibitory Effects of Scopoletin in Collagen-induced Human Platelet Aggregation

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INTRODUCTION

Platelet aggregation is essential for the formation of a hemostatic plug when normal blood vessels are injured. However, the interactions between platelets and collagen can also cause circulatory disorder such as thrombosis, atherosclerosis, and myocardial infarction [1]. Collagen supports platelet adhesion to the sub-endothelium and induces subsequently aggregation, secretion, and procoagulant activity. Therefore, inhibition of the platelet-collagen interaction might be a promising approach to the prevention of thrombosis. An important role in collagen-
induced mechanisms of platelet aggregation is mediated by the formation of thromboxane A2 (TXA2) [2], which contributes to an increase in intracellular Ca\(^{2+}\) mobilization ([Ca\(^{2+}\)]\(_i\)) in collagen–activated platelets. An increase in [Ca\(^{2+}\)]\(_i\) activates both the Ca\(^{2+}\)/calmodulin dependent phosphorylation of myosin light chain (MLC) and the diacylglycerol-dependent phosphorylation of cytosolic pleckstrin to induce platelet aggregation [3, 4]. In addition, diacylglycerol can also be hydrolyzed by diacylglycerol and monoacylglycerol lipase to produce arachidonic acid, a precursor of TXA2, which is a potent platelet aggregation agent generated from arachidonic acid [3–5]. Autocrine agonists, such as adenosine diphosphate (ADP) and TXA2, have been reported to be involved in platelet secretion and aggregation induced by low concentrations of collagen [6].

Verapamil and theophylline have an antiplatelet function by elevating the level of cyclic adenosine monophosphate (cAMP) that decrease the [Ca\(^{2+}\)]\(_i\), an essential factor for platelet aggregation. Vasodilators (such as molsidomine and nitroprusside) and cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE) inhibitors [such as zaprinast and erythro-9-(2-hydroxy-3-nonyl) adenine] elevate cGMP levels in platelets [5]. The antiplatelet effects of cAMP and cGMP are mediated by cAMP and cGMP-dependent protein kinases (A-kinase and G-kinase, respectively) that phosphorylate the substrate protein vasodilator-stimulated phosphoprotein (VASP) [7, 8]. VASP is an actin-binding protein and plays an important role in negatively regulating secretion and adhesion [8], and phosphorylation of VASP is known to be associated with inhibition of VASP affinity for contractile protein filamentous actin as well as \(\alpha/\beta_3\) [9].

Scopoletin, which is found commonly in the root of plants in the genus Scopolia or Artemisia, is known to have anti-malaria and anti-coagulation effects [10, 11]. However, little is known regarding the role of scopoletin in platelet aggregation, and the mechanism of scopoletin on collagen-induced human platelet aggregation. In order to clarify the mode of antithrombotic action of scopoletin, we investigated the effect of scopoletin on various parameters associated with collagen-induced platelet aggregation.

**MATERIALS AND METHODS**

1. **Materials**

Scopoletin was obtained from Avention Corporation (Seoul, Korea) (Figure 1). Collagen was obtained from Chrono-Log Corporation (Havertown, PA, USA). LDH Cytotoxicity assay kit, and TXB\(_2\), ATP, cAMP and cGMP enzyme immunoassay (EIA) kits were bought from Cayman Chemical (Ann Arbor, MI, USA). Fura 2-acetoxymethyl ester (2-AM) and fibrinogen Alexa Fluor 488 conjugate were obtained from Invitrogen (Eugene, OR, USA).

2. **Preparation of human washed platelets**

Human platelet-rich plasma (PRP) was obtained from Korean Red Cross Blood Center (Suwon, Korea). Washed platelets (10\(^8\) platelets/mL) were prepared according to previously published methods [12]. PRP was centrifuged at 1,300 G for 10 minutes to obtain platelet pellets. This was washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO\(_3\), 0.36 mM NaH\(_2\)PO\(_4\), 5.5 mM glucose, and 1 mM EDTA, pH 6.9). The washed platelets were resuspended in suspension buffer (138 mM NaCl, 5.5 mM glucose, 2.7 mM KCl, 12 mM NaHCO\(_3\), 0.36 mM NaH\(_2\)PO\(_4\), 0.49 mM MgCl\(_2\), and 0.25% gelatin, pH 7.4) to a final concentration of 10\(^8\) platelets/mL. All of the procedures were carried out at 25\(^\circ\)C to avoid platelet aggregation at low temperature. The experimental use was approved by the Institutional Review Board of the Namseoul University (1041479-HR-201803-003).

3. **Measurement of cytotoxicity**

Cytotoxicity was determined through the leakage of

![Figure 1. The structure of scopoletin.](www.kjcls.org)
Human washed platelets ($10^8$ platelets/mL) were incubated for 2 hours at room temperature with various concentrations of scopoletin and then centrifuged at room temperature for 2 minutes at 12,000 G. The supernatant was measured by LDH cytotoxicity assay kit (Cayman Chemical) at an optical density of 490 nm. The cytotoxicity is expressed as the cell viability through the percentage of the total enzyme activity in platelets completely lysed with 0.1% Triton X-100.

4. Measurement of platelet aggregation and TXB$_2$

The washed platelets ($10^8$ platelets/mL) were preincubated for 3 minutes at 37°C with substances, and external 2 mM CaCl$_2$ was added. After that, the washed platelets stimulated with collagen (2.5 μg/mL) for 5 minutes. The aggregation was monitored using an aggregometer (Chrono-Log Co., Havertown, PA, USA) at 1,000 rpm. Each aggregation rate was evaluated as increase in light transmission. The reactions were stopped by the addition of ice-cold EDTA (5 mM) and indomethacin (0.2 mM). The amount of TXB$_2$, a stable metabolite of TXA$_2$, was determined using a TXB$_2$ EIA kit (Cyanam Chemical).

5. Measurement of cytosolic Free Ca$^{2+}$ ([Ca$^{2+}$]).

PRP was incubated with 5 μM Fura 2-AM for 60 minutes at 37°C. Because Fura 2-AM is light sensitive, the tube containing the PRP was covered with aluminum foil. The Fura 2-loaded washed platelets were prepared using the procedure described above and 10$^8$ platelets/mL were preincubated for 3 minutes at 37°C with or without scopoletin in the presence of 2 mM CaCl$_2$ and then stimulated with collagen (2.5 μg/mL) for 5 minutes. The fluorescence of Fura 2 was measured with a spectrofluorometer (SFM 25, BioTek Instrument, Italy), and the [Ca$^{2+}$]$_i$ values were calculated using the method of a research [13].

6. Measurement of cyclic nucleotides (cAMP and cGMP)

The washed platelets ($10^8$ platelets/mL) were preincubated for 3 minutes at 37°C with or without various concentrations of scopoletin in the presence of 2 mM CaCl$_2$ and then stimulated with collagen (2.5 μg/mL) for 5 minutes for platelet aggregation. The aggregation was terminated by the addition of 1M HCl. cAMP and cGMP were measured by using Synergy HT Multi-Model Microplate Reader (BioTek Instruments, Winooski, VT, USA).

7. Measurement of fibrinogen binding to αIib/β3

The platelet aggregation assay was conducted at 37°C for 5 min, with Alexa Flour 488-human fibrinogen (30 μg/mL) binding platelets. To terminate the reaction, 0.5% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) was added. The mentioned procedures were conducted in darkness. The fibrinogen binding assay was performed using flow cytometry (BD Biosciences, San Jose, CA, USA), and analyzed with the CellQuest software (BD Biosciences).

8. Measurement of ATP release

The washed platelets ($10^8$ platelets/mL) were preincubated for 3 minutes at 37°C with or without various concentrations of scopoletin in the presence of 2 mM CaCl$_2$ and then stimulated with collagen (2.5 μg/mL) for 5 minutes. The reaction was terminated and centrifuged with 1,000 G at 4°C for 10 minutes, and the supernatant was used for ATP release assay. ATP release was measured with an ATP assay kit in a luminometer (BioTek Instruments).

9. Statistical analysis

The results are expressed as means±SD. Statistical analysis was performed with a two tailed-unpaired Student’s t-test or ANOVA, as appropriate. If there were significant differences between the group means according to ANOVA, each group was compared by Scheffe’s method.
RESULTS

1. Effects of scopoletin on platelet aggregation and cytotoxicity

As shown in Figure 2A, the platelet aggregation induced by collagen was 98.0±2.0%, but scopoletin (50, 100, 300 and 500 μM) significantly reduced platelet aggregation by 89.0±5.3, 64.3±7.4, 47.3±7.6 and 16.3±3.6%, respectively. In addition, the cytotoxicity of scopoletin to human platelets was confirmed to have no significant effects (Figure 2B). This means that it is valuable as a platelet aggregation inhibitor.

2. Effects of scopoletin on TXB2 and [Ca2+]

The amount of TXB2 in intact platelets was 2.4±1.1 ng/10⁸ platelets, and this was increased to 185.9±13.4 μg/10⁸ platelets when platelets were stimulated with collagen (2.5 μg/mL). However, scopoletin (100, 300 and 500 μM) significantly reduced the levels of TXB2 in dose–dependent (Figure 3A). As shown in Figure 3B, scopoletin (50, 100, 300 and 500 μM) strongly inhibited [Ca2+] increased by collagen.

3. Effects of scopoletin on cyclic nucleotides

Both cAMP and cGMP are known to be negative regulators of platelet aggregation due to a decrease in [Ca2+] levels [14]. Therefore, we investigated whether scopoletin affects cellular levels of cAMP or cGMP. As shown in Figure 4A, scopoletin strongly increased cAMP levels from 3.9±0.4 pmol/10⁸ platelets to 10.2±0.5 pmol/10⁸ platelets. On the other hand, although cGMP was increased by scopoletin from 6.5±0.5 pmol/10⁸

Figure 2. Effects of scopoletin on platelet aggregation. (A) Effects of scopoletin on platelet aggregation stimulated by collagen. (B) Effects of scopoletin on cytotoxicity. Data are expressed as mean±SD (N=4). * or ** mean P<0.05 or P<0.001 compared with the collagen–stimulated platelets.

Figure 3. Effects of scopoletin on TXB2 production and [Ca2+]. (A) Effects of scopoletin on TXA2 production stimulated by collagen. (B) Effects of scopoletin on [Ca2+], stimulated by collagen. Data are expressed as mean±SD (N=4). * means P<0.05 compared with no–stimulated platelets, ** or *** means P<0.05 or P<0.001 compared with the collagen–stimulated platelets.
platelets to 7.8±0.2 pmol/10⁸ platelets, it was not as strong as cAMP (Figure 4B). These results indicate that scopoletin upregulates the production of cAMP and cGMP in collagen-stimulated platelets.
4. Effects of scopoletin on fibrinogen binding to $\alpha_{\text{IIb/}}/\beta_3$

Increased nucleotides are known to inhibit fibrinogen binding to $\alpha_{\text{IIb/}}/\beta_3$ complex, which are concerned with inhibition of platelet activation [15, 16]. Therefore, we examined whether scopoletin inhibits fibrinogen binding to $\alpha_{\text{IIb/}}/\beta_3$. As shown in Figure 5A and 5B, collagen increased potently fibrinogen binding to $\alpha_{\text{IIb/}}/\beta_3$ from 1.2±0.1% to 90.8±0.5%. However, scopoletin inhibited dose-dependently collagen-induced fibrinogen binding to $\alpha_{\text{IIb/}}/\beta_3$ from 90.8±0.5% to 18.2±0.6%.

5. Effects of scopoletin on ATP

As shown in Figure 6, ATP level in supernatant from the collagen-induced platelets was 8.3±0.1 $\mu$M, which is 41.5 fold as compared with that (0.2±0.1 $\mu$M) in intact cell. However, scopoletin (50, 100, 300 and 500 $\mu$M) inhibited dose-dependently collagen-elevated ATP release from 8.3±0.1 $\mu$M to 1.2±0.1 $\mu$M, and scopoletin (500 $\mu$M) inhibited potently ATP release to 85.5% as compared with collagen-induced ATP.

DISCUSSION

During platelets activation, PLC-γ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) with IP3 and diacylglycerol (DAG). In addition, IP3 causes $Ca^{2+}$ mobilization from the endoplasmic reticulum, and DAG activates DAG-dependent protein kinase C [17]. Increased [$Ca^{2+}$] results to phosphorylation of myosin light chain (20-kDa) and cytosolic protein (40- or 47-kDa) through $Ca^{2+}$/calmodulin-dependent phosphorylation, which lead to induce platelet aggregation [3]. Thromboxane A2 (TXA2) is a potent vasoconstrictor that stimulates platelet aggregation and subsequent platelet activation. TXA2 is finally produced from arachidonic acid through PGH2 by the action of enzymes such as cyclooxygenase (COX) and thromboxane A synthase (TXAS) [18]. It is known that the reduction of TXA2 is essential for the antithrombotic process by inhibiting platelets activation. In this study, scopoletin suppressed TXA2 production and [$Ca^{2+}$] in human platelet dose-dependently, as the result, inhibit platelet aggregation.

Cyclic nucleotides are known to reduce $Ca^{2+}$ influx and inhibit platelet aggregation through cAMP- and cGMP-dependent protein kinases [19]. In this study, scopoletin increased cAMP- and cGMP production in platelets. These results indicate that the increase of cyclic nucleotides by scopoletin can play a central role in platelet aggregation. The cAMP and cGMP are dependent on the activation of adenyl cyclase/guanyl cyclase or cyclic nucleotide phosphodiesterases (PDEs) [20]. Inhibition of PDE activity increases the level of cyclic nucleotides in platelet aggregation [21]. Thus, PDE inhibitors may have therapeutic potential for thrombosis. Indeed, PDE inhibitors such as triflusal, cilostazol and dipyridamole have been used clinically as antiplatelet drugs to increase the production of cyclic nucleotides [5, 22]. Therefore, scopoletin might affect the activity of cyclic nucleoside phosphodiesterase, and further studies are required to reveal this. In any case, scopoletin increased the production of the cytoplasmic nucleosides such as cAMP and cGMP, especially cAMP increased potently.

Increased cAMP production in platelets has been reported to inhibit platelet activation via VASP ser157 phosphorylation [23, 24]. VASP is a major substrate for cAMP-dependent PKA, and stimulation of VASP inhibits platelet activation by modulating the secretory and
adhesive properties of platelets [25]. Moreover, VASP phosphorylation also inhibits the activation of integrin αIIb/β3, which consequently inhibits platelet aggregation. In our study, scopoletin significantly suppressed fibrinogen binding to αIIb/β3 induced by collagen, which may be due to the elevation of cAMP production. These results indicate that scopoletin have therapeutic potential against platelet-related disorders, which might be related to cyclic nucleotides-downstream pathway. In addition, the increase of ATP release from dense body in platelets is known to be involved in amplification of platelet aggregation [26, 27]. Because scopoletin inhibited ATP release dose-dependently, it is thought to have helped inhibit aggregation amplification. These results suggest that scopoletin, one of the phytochemical compounds, may have an inhibitory effect on collagen-induced human platelet aggregation by regulating aggregation-inducing molecules (TXA2 and Ca²⁺) and aggregation-inhibiting molecules (cAMP and cGMP).

In conclusion, we have presented many evidences that scopoletin is an effective antiplatelet agent. This study suggests that scopoletin has a preventive effect on platelet derived vascular thrombosis.

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