Antiplatelet effects of scoparone through up-regulation of cAMP and cGMP on U46619-induced human platelets

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Abstract Platelet activation is essential for hemostatic process on blood vessel damage. However, excessive platelet activation can cause some cardiovascular diseases including atherosclerosis, thrombosis, and myocardial infarction. Scoparone is commonly encountered in the roots of genus *Artemisia* or *Scopolia*, and has been studied for its potential pharmacological properties including immunosuppression and vasorelaxation, but antiplatelet effects of scoparone have not been reported yet. We investigated the effect of scoparone on human platelet activation prompted by an analogue of thromboxane A₂, U46619. As the results, scoparone dose-dependently increased cyclic adenosine monophosphate (cAMP) levels as well as cyclic guanosine monophosphate (cGMP) levels, both being aggregation-inhibiting molecules. In addition, scoparone strongly phosphorylated inositol 1, 4, 5-triphosphate receptor (IP₃R) and vasodilator-stimulated phosphoprotein (VASP), substrates of cAMP dependent kinase and cGMP dependent kinase. Phosphorylation of IP₃R by scoparone resulted in inhibition of Ca²⁺ mobilization in calcium channels in a dense tubular system, and phosphorylation of VASP by scoparone led to an inability of fibrinogen being able to bind to α IIb/ β 3. Finally, scoparone inhibited thrombin-induced fibrin clotting, thereby reducing thrombus formation. Therefore, we suggest that scoparone has a strong antiplatelet effect and is highly probable to prevent platelet-derived vascular disease.

Keywords Cyclic adenosine monophosphate/Cyclic guanosine monophosphate · Fibrinogen binding · Inositol 1, 4, 5-triphosphate receptor · Intracellular Ca²⁺ · Scoparone · Vasodilator-stimulated phosphoprotein

Introduction

Platelet aggregation is an essential response to the formation of hemostatic plug when blood vessels are damaged. In particular, the concentration of Ca²⁺ ([Ca²⁺]_i) in the cytoplasm of platelets plays a key role in the activation of platelet aggregation. [Ca²⁺]_i is known to be recruited into the cytoplasm through the receptors from the dense tubular system, an internal reservoir, by inositol 1, 4, 5-triphosphate (IP₃) [1]. Increased [Ca²⁺]_i phosphorylates myosin light chain (20 kDa), which is dependent on the Ca²⁺/calmodulin complex, and phosphorylates pleckstrin by activating protein kinase C in cooperation with diacylglycerol (DG). This leads to rearrangement of cytoskeletal proteins and finally to platelet aggregation [1].

In addition, DG, which is produced by the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP₂) located on the platelet membrane, is sequentially hydrolyzed by DG lipase and monoacylglycerol lipase and converted to Thromboxane A₂ (TXA₂) via arachidonic acid [2]. TXA₂ is known to activate platelets to induce secretion and morphological changes [3]. Indeed, U46619 (9,11-dideoxy-9 α ,11 α -methanoepoxyprostaglandin F_{2a}), a stable analogue of TXA₂, is used as a proper platelet aggregation inducer to increase phosphorylation of the myosin light chain and pleckstrin by increasing [Ca²⁺]_i [4,5]. This is a normal process of hemostatic reactions in the blood but can also cause various vascular diseases such as atherosclerosis when excessive platelet aggregation occurs. Thus, adequately inhibiting platelet aggregation may be a useful approach to prevent cardiovascular disease [4,5].

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Verapamil and theophylline function as antiplatelets by increasing levels of cyclic adenosine monophosphate (cAMP), which reduces $[Ca^{2+}]_i$, an essential component of platelet aggregation. In addition, guanosine monophosphate (cGMP) levels in platelets are increased with the use of cyclic cGMP phosphodiesterase (PDE) inhibitors like erythro-9- (2-hydroxy-3-nonyl) adenine and zaprinast, or vasodilators such as molsidomine and nitroprusside [6]. During normal circulation, vascular endothelial cells release nitric oxide and prostaglandin I_2 , which causes the platelets to produce cyclic adenosine monophosphate (cAMP) and cyclic cGMP. Elevated cAMP levels induce activation of protein kinase A (PKA) while elevated cGMP levels lead to the activation of protein kinase G (PKG). Together, PKA and PKG phosphorylate substrate protein inositol 1, 4, 5-triphosphate receptor (IP_3R) and vasodilator-stimulated phosphoprotein (VASP) [7]. Phosphorylation renders IP_3R inactive, causing mobilization of $[Ca^{2+}]_i$ to become inhibited [8,9]. In the platelet, vasodilator-stimulated phosphoprotein is a major substrate of PKA and PKG and it contributes to activation of $\alpha IIb/\beta_3$ as well as the regulation of actin filament dynamics. However, suppression of $\alpha IIb/\beta_3$ activation and inhibition of actin filament elongation can be caused through cAMP-dependent VASP Ser¹⁵⁷ phosphorylation or cGMP-dependent VASP Ser239 phosphorylation [10,11]. As a result, phosphorylation of IP_3R can be very helpful when investigating the Ca^{2+} -antagonistic effect, whereas VASP phosphorylation is essential for evaluating $\alpha IIb/\beta_3$ -inhibition effect.

Scoparone, commonly found in the roots of plants of the genus *Scopolia* or *Artemisia*, has been studied for its potential

pharmacological properties including immunosuppression and vasorelaxation [12,13]. However, the role of scoparone in platelet activation and the mechanism of scoparone on human platelet aggregation caused by U46619 is unknown. This study examined the effect of scoparone on various factors related to U46619-induced platelet activation to clarify the antiplatelet effects of scoparone.

Materials and Methods

Materials

Scoparone (6,7-Dimethoxy-2H-chromen-2-one) was purchased from Avention Corporation (Seoul, Korea) (Fig. 1). Chrono-Log Corporation (Havertown, PA, USA) provided U46619. Both cAMP and cGMP enzyme immunoassay (EIA) kits were received from Cayman Chemical (Ann Arbor, MI, USA). Fura 2-AM and Fibrinogen Alexa Fluor 488 conjugates were purchased from Invitrogen (Eugene, OR, USA). Anti-VASP, anti-phosphor-VASP Ser²³⁹, anti-phosphor-VASP Ser¹⁵⁷, anti-phosphor-inositol-3-phosphate receptor type I, the lysis buffer, anti-rabbit IgG-HRP-conjugate, and anti- β -actin were obtained from Cell Signaling (Beverly, MA, USA). Enhanced chemiluminescence solution (ECL) and polyvinylidene difluoride (PVDF) membrane were purchased from General Electric Healthcare (Chalfont St. Giles, Buckinghamshire, UK). The Fibrinogen Alexa Fluor 488 conjugate was obtained through Invitrogen Molecular Probes (Eugene, OR, USA).

Fig. 1 The structure of scoparone and the effects of scoparone on U46619-induced platelet aggregation. (A) Chemical structure of scoparone. PIN: 6,7-Dimethoxy-2H-chromen-2-one, Chemical formula: $C_{11}H_{10}O_4$, Molar mass: 206.19 g/mol. (B) Effects of scoparone pretreatment on U46619-stimulated platelet aggregation. (C) Cytotoxicity of scoparone on human platelets. Washed platelets (10^8 /mL) were preincubated with various concentrations of scoparone in the presence of 2 mM $CaCl_2$ for 3 min at 37 °C, then stimulated with U46619 (0.5 μ M) for 5 min. Platelet aggregation (%) was recorded as an increase in light transmission. The data are expressed as the mean $\pm$ standard deviation (n=4). * p < 0.05, ** p < 0.001 compared with the U46619-stimulated platelets

Preparation of washed human platelets

Korean Red Cross Blood Center (Suwon, Korea) provided human platelet rich plasma (PRP). Washed platelets were prepared according to the previously published method [14]. PRP was centrifuged for 10 min at $1,300\times g$ to obtain platelets, which were washed twice with wash buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO_3 , 0.36 mM NaH_2PO_4 , 5.5 mM glucose, and 1 mM Na_2EDTA , pH 6.9). A suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO_3 , 0.36 mM NaH_2PO_4 , 0.49 mM MgCl_2 , 5.5 mM glucose, 0.25% gelatin, pH 7.4) at a final concentration of $10^8/\text{mL}$ was used to suspended the washed platelets. All procedures were performed at 25°C . to avoid platelet aggregation at low temperatures. This experiment was performed and executed with the approval of the Institutional Review Board of Namseoul University (1041479-HR-201803-003).

Platelet aggregation

Platelets (10^8 cells/mL) were preincubated in separate samples with or without scoparone in 2 mM CaCl_2 for 3 min at 37°C , and U46619 (0.5 μM) was then added to stimulate. An aggregometer was used for 5 min to perform the platelet aggregation assay. An increase in light transmission was used to determine the platelet aggregation rate (%).

Measurement of cytotoxicity

Cytotoxicity was determined through the leakage of lactate dehydrogenase (LDH) from cytosol. Human washed platelets (10^8 cells/mL) were incubated for 2 hours at room temperature with various concentrations of substances and then centrifuged at room temperature for 2 min at 12,000 g. The supernatant was measured by LDH assay kit at an optical density of 490 nm.

Intracellular calcium concentration

For 60 min, PRP was incubated at 37°C with 5 μM of Fura 2-AM. Fura 2-AM is sensitive to light, so aluminum foil was used to cover the tube containing PRP. Washed platelets (10^8 cells/mL) filled with Fura 2 were prepared using the procedure specified above, incubated at 37°C together with 2 mM CaCl_2 for 3 min, and then treated for 5 min by treatment with U46619 (0.5 μM). The fluorescence of Fura 2 was measured using a spectrophotometer (SFM 25, BioTeck Instrument, Legnano, Italy). The excitation wavelength was initially set at 340 nm and increased at increments of 0.5 s ultimately reaching 380 nm. A wavelength setting of 510 nm was used for the emission wavelength. The calculations of $[\text{Ca}^{2+}]_i$ values were performed using the Grynkiewicz method [15].

Immunoblotting

Platelet aggregation was terminated by addition of $1\times$ lysis buffer. The resulting protein concentration of the platelet lysates was measured and evaluated using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Proteins (20 μg) were

Fig. 2 Effects of scoparone on cyclic nucleotides production. (A) Effects of scoparone on cAMP production stimulated by U46619. (B) Effects of scoparone on cGMP production stimulated by U46619. Washed platelets ($10^8/\text{mL}$) were preincubated with or without scoparone for 3 min in the presence of 2 mM CaCl_2 and then stimulated with U46619 (0.5 μM) for 5 min at 37°C . The reactions were terminated by adding 1 M HCl. cAMP and cGMP contents were measured using EIA kits. The data are expressed as the mean $\pm$ standard deviation ($n=4$). * $p < 0.05$, ** $p < 0.001$ compared with the U46619-stimulated platelets

separated through 4-20% SDS-PAGE, and relocated onto PVDF membranes. The primary antibodies were set at a dilution of 1:1,000 and the secondary antibodies were set at a dilution of 1:10,000. The protein bands were visualized using ECL reagent (General Electric Healthcare, Buckinghamshire, UK).

Fibrinogen binding to $\alpha\text{IIb}/\beta_3$

Washed platelets (10^8 cells/mL) bound to Alexa Flour 488-human fibrinogen (30 $\mu\text{g}/\text{mL}$) were stimulated with U46619 (0.5 μM) after adding 2 mM CaCl_2 . To terminate the reaction, phosphate-buffered saline (PBS, pH 7.4) containing 0.5% paraformaldehyde was added. This process was performed while blocking the light, fibrinogen binding capacity was performed using a flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed by Cell-Quest software (BD Biosciences).

Platelet-mediated fibrin clot retraction

Human PRP (500 μL) was moved into a polyethylene tube to

Fig. 3 Effects of scoparone on IP₃R phosphorylation and [Ca²⁺]_i mobilization. (A) Effects of scoparone on U46619-induced IP₃R phosphorylation. (B) Inhibitory effects of scoparone on U46619-induced [Ca²⁺]_i mobilization. Fura 2-filled washed platelets (10⁸ cells/mL) were incubated at 37 °C with 2 mM CaCl₂ for 3 min, and then treated for 5 min with U46619 (0.5 μ M). The fluorescence of Fura 2 was measured using a spectrophotometer (SFM 25, BioTeck Instrument, Italy). The excitation wavelength was initially set at 340 nm and increased at increments of 0.5 s ultimately reaching 380 nm. A wavelength setting of 510 nm was used for the emission wavelength. 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 U46619-stimulated platelets

avoid adhesion, various levels of scoparone (10, 30, 50 μ M) were preincubated with separate samples for 15 min at 37 °C, using thrombin (0.05 U/mL) for stimulation. At 15 min, pictures of fibrin clot were taken using a digital camera. The area of the clot was calculated using the Image J Software (v1.46, National Institutes of Health, Bethesda, MD, USA).

Statistical analyses

Mean $\pm$ standard deviation was used to express the experimental results, and statistical analysis was appropriately performed using either unpaired Student's *t*-test or ANOVA. Groups were compared by Scheffe's method if there were significant differences between group means according to ANOVA.

Results

Effects of scoparone on U46619-induced human platelet aggregation

U46619 at 0.5 μ M induces the optimum aggregation of human platelets, and in this study, U46619-induced platelets aggregation rate was 80.5% (Fig. 1B). However, platelets treated with scoparone (5, 10, 30, and 50 μ M) had aggregation rates that were significantly reduced (6.5, 40.4, 85.1, and 93.8%, respectively) without cytotoxicity (Fig. 1C), which indicates that scoparone inhibited U46619-induced platelet aggregation in a dose-dependent manner.

Effects of scoparone on elevation of cAMP and cGMP

We confirmed whether scoparone affects the production of cAMP or cGMP. As shown in Fig. 2A scoparone strongly increased cAMP production from 4.23 $\pm$ 0.16 to 6.26 $\pm$ 0.21 pmol/10⁸ cells. In addition, cGMP was significantly increased by scoparone from 5.92 $\pm$ 0.38 to 10.41 $\pm$ 0.91 pmol/10⁸ cells (Fig. 2B). These results indicate that scoparone functions in suppressing platelets activation by increasing the production of cAMP and cGMP in U46619-induced platelets.

Effects of scoparone on elevation of [Ca²⁺]_i mobilization, IP₃RI phosphorylation

Resulting from the fact that [Ca²⁺]_i is crucial for platelet activation, we examined the effect of scoparone on Ca²⁺ antagonistic activity. As shown in Fig. 3A, [Ca²⁺]_i level was raised from 100.9 $\pm$ 0.5 nM to 609.5 $\pm$ 7.2 nM by U46619. However, scoparone dose (10 to 50 μ M)-dependently decreased collagen-elevated [Ca²⁺]_i level (Fig. 3A). The inhibition rate of scoparone (50 μ M) was 79.5%. Therefore, we expected that scoparone affects calcium mobilization and assessed the calcium mobilization signaling molecule and the effect of its phosphorylation. As shown in Fig. 3C, scoparone (10 to 50 μ M) dose-dependently increased IP₃RI (Ser¹⁷⁵⁶) phosphorylation in U46619-induced human platelet aggregation. This demonstrates that the decrease of intracellular calcium level by scoparone resulted from IP₃RI (Ser¹⁷⁵⁶) phosphorylation.

Fig. 4 Effects of scoparone on VASP phosphorylation. Immunoblotting was conducted to analyze the phosphorylation of signaling molecules extracted from the lysates of U46619-stimulated washed platelets that were pre-treated with scoparone. Representative immunoblot images and data (mean±SD) 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 U46619-stimulated platelets

Effects of scoparone on VASP-phosphorylation

As scoparone showed a dose-dependent increasing effect on U46629-induced cAMP and cGMP level (Fig. 2), we further investigated the effect of scoparone on cAMP-dependent VASP Ser¹⁵⁷ phosphorylation and cGMP-dependent VASP Ser²³⁹ phosphorylation in U46619-activated platelets. Scoparone significantly increased VASP Ser¹⁵⁷ and VASP Ser²³⁹ phosphorylation (Fig. 4) in a dose-dependent manner.

Effects of scoparone on fibrinogen binding to αIIb/β₃

Since scoparone increased VASP Ser¹⁵⁷ phosphorylation and VASP Ser²³⁹ phosphorylation through elevation of cAMP and cGMP production (Fig.4), we investigated the effect of scoparone on fibrinogen binding to αIIb/β₃. U46619 increased the fibrinogen binding to αIIb/β₃ (Fig. 5A-b, 5B), with a rate of 91.7±1.0%. However, fibrinogen binding was attenuated with the use of scoparone in a dose-dependent manner (Figs. 5A-c-f, 5B). The inhibition rate of scoparone (50 μM) was 81.3%.

Effects of scoparone on fibrin clot retraction

The binding of fibrinogen to αIIb/β₃ is increased by the activation of αIIb/β₃ through platelet agonists. In time, prompting the outside-in αIIb/β₃ signaling pathway as well as clot retraction.

Therefore, we investigated thrombin-induced fibrin clot retraction, an index of outside-in αIIb/β₃ signaling, in relation to the effect of scoparone. Fig. 6A displays both thrombin stimulated fibrin clot formation and clot retraction. Yet scoparone (10, 30 and 50 μM) suppressed the thrombin-retracted fibrin clot, and these inhibitory degrees were 14.9, 58.5, and 92.2%, respectively as compared with that by thrombin (Fig. 6B).

Discussion

When platelet activation occurs, phospholipase C-γ₂ hydrolyzes PIP₂ to IP₃ and diacylglycerol (DG) on the platelet membrane. The resulting IP₃ induces intracellular Ca²⁺ ([Ca²⁺]_i) mobilization from the dense tubular system, and DG-dependent protein kinase C is activated through DG [16]. Increased [Ca²⁺]_i causes phosphorylation of Ca²⁺/calmodulin dependent proteins myosin light chain (20 kDa) and pleckstrin (40 or 47 kDa) resulting in platelet activation [17].

On the other hand, cyclic nucleotides is known to inhibit platelet aggregation by reducing [Ca²⁺]_i and activating cAMP- and cGMP-dependent protein kinase (PKA and PKG) [18]. In this study, scoparone significantly inhibited U46619-induced platelets aggregation without cytotoxicity (Fig. 1). In addition, scoparone increased cAMP and cGMP production in platelets in a concentration-dependent manner, and suppressed [Ca²⁺]_i concentrations. These results indicate that increased scoparone-induced cyclic nucleotides played a central role in inhibiting platelet activity by down-regulating [Ca²⁺]_i. Increased cAMP and cGMP can be used to phosphorylate multiple substrates by activating PKA and PKG, of which it is known to affect inositol 1, 4, 5-triphosphate receptor (IP₃R) phosphorylation [7]. As shown in Fig. 3A, the phosphorylation of IP₃R was increased in a concentration-dependent manner by scoparone. This suggests that scoparone inhibited [Ca²⁺]_i by inhibiting the opening of calcium channels in the dense tubular system through phosphorylation of IP₃R, which appears to be due to the activation of PKA and PKG. In addition, the increase of cAMP and cGMP production by scoparone resulted in the phosphorylation of VASP protein, a substrate through activation of PKA and PKG. VASP acts as a major substrate of cAMP/cGMP-dependent PKA/PKG that regulates platelet activation by regulating platelet secretion and adhesion properties, and VASP phosphorylation is also known to inhibit activation of integrin αIIb/β₃ and consequently inhibit platelet aggregation [19,20].

In this study, scoparone strongly inhibited fibrinogen binding to αIIb/β₃ in U46619-stimulated platelets. This is believed to be related to VASP Ser¹⁵⁷ and VASP Ser²³⁹ phosphorylation due to increased cAMP and cGMP production by scoparone. In addition, Integrin αIIb/β₃-mediated signal transduction usually leads to deformation of the platelet cytoskeleton, which affects platelet proliferation and coagulation regression. Coagulation contraction is the most important step during repair of damaged parts of blood

Fig. 5 Effects of scoparone on U46619-induced fibrinogen binding. (A) The flow cytometry histograms on fibrinogen binding. a, Intact platelets (base); b, U46619 (0.5 μ M); c, U46619 (0.5 μ M) + scoparone (5 μ M); d, U46619 (0.5 μ M) + scoparone (10 μ M); e, U46619 (0.5 μ M) + scoparone (30 μ M); f, U46619 (0.5 μ M) + scoparone (50 μ M). (B) Effects of scoparone on U46619-induced fibrinogen binding (%). Washed platelets (10^8 cells/mL) bound to Alexa Flour 488-human fibrinogen (30 μ g/mL) were stimulated with U46619 (0.5 μ M) after adding 2 mM CaCl_2 . Fibrinogen binding capacity was performed using a flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed by Cell-Quest software (BD Biosciences). The data are expressed as the mean $\pm$ standard deviation ($n=4$). * $p < 0.05$ compared with no-stimulated platelets, ** $p < 0.001$ compared with the U46619-stimulated platelets

vessels. Damaged blood vessels accumulate activated platelets and develop into fibrin platelet meshwork. Solidification formation, sealing the damaged container, begins to shrink for a time of about 30 to 60 min and pulls the cutting edges together. The interaction between $\alpha\text{IIb}/\beta_3$ and fibrin plays an important role in coagulation formation, and $\alpha\text{IIb}/\beta_3$ inhibitors strongly inhibit coagulation contraction [21]. Thrombin causes coagulation and activates platelet $\alpha\text{IIb}/\beta_3$, which increases the binding of fibrinogen to $\alpha\text{IIb}/\beta_3$, causing coagulation shrinkage. As shown in Fig. 6, the antiplatelet effect of scoparone suppressed thrombin-induced fibrin coagulation, which is the actual result of thrombosis inhibition. These results suggest that scoparone has potential as a potent antiplatelet substance that increases cAMP and cGMP production and inhibits thrombogenesis.

Further research is needed on which mechanisms have increased the production of cAMP and cGMP. Some previous studies have reported that scoparone promotes penile erection through vasodilation by increasing nitric oxide and increasing cGMP

production [22]. We also confirmed that scoparone strongly increased cGMP production, and this result could be attributed to the effect of NO. In addition, cAMP and cGMP rely on the activation of cyclic nucleotide PDE or adenylyl cyclase/guanylyl cyclase [23]. Since cyclic nucleotide levels in platelet aggregation is increased through the inhibition of PDE activity, PDE inhibitors may have a therapeutic effect on thrombosis [24]. Indeed, PDE inhibitors such as triple rasul, cilostazol and dipyridamole are used as antiplatelets to clinically increase the production of cyclic nucleotides [6]. Scoparone may be developed as a drug with antiplatelet action under similar mechanism.

In conclusion, scoparone phosphorylated VASP and IP_3R by increasing cAMP and cGMP in human platelets, inhibiting the activation of integrin $\alpha\text{IIb}/\beta_3$ and the recruitment of Ca^{2+} into the cytoplasm. As a result, fibrin coagulation of thrombin induction was strongly inhibited. Therefore, this study suggests that scoparone can be developed as an effective antithrombotic and therapeutic agent.

Fig. 6 Effects of scoparone on fibrin clot retraction. (A) Effects of scoparone on thrombin-retracted fibrin clot photographs (B) Effects of scoparone on thrombin-retracted fibrin clot area. Human PRP (500 µL) was moved into a polyethylene tube to avoid adhesion, various scoparone (10, 30, 50 µM) were preincubated with separate samples for 15 min at 37 °C, using thrombin (0.05 U/mL) for stimulation. At 15 min, pictures of fibrin clot were taken using a digital camera. The area of the clot was calculated using the Image J Software (v1.46, National Institutes of Health, USA). 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 U46619-stimulated platelets

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Conflict of interest The authors declare no conflict of interest.

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