Natural Coumarin Derivative Esculetin Regulates Platelet Activation via Modulating NF-κB Signaling in Cyclic Nucleotide-Independent Manner

Chih-Wei Hsia1*, Kou-Gi Shyu1,2*, Thanasekaran Jayakumar1, Chih-Hsuan Hsia1,3, Marappan Velusamy4, Chih-Hao Yang1,5, and Joen-Rong Sheu1,5

Abstract

Esculetin, a natural coumarin derivative, shows exciting biological activities in a variety of cell and animal models. Our recent study demonstrated that esculetin exhibits antiplatelet effects by obstructing the phospholipase C γ2/protein kinase C cascade, hydroxyl radical formation, and Akt activation. In this study, we further examined the involvement of cyclic 3′-5′adenosine monophosphate/, vasodilator-stimulated phosphoprotein (VASP), integrin αIIbβ3, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), since cyclic nucleotides reduce the phosphorylation of VASP and activate NF-κB, subsequently inducing αIIbβ3 activation that significantly involves the platelet inhibitory pathways. We found that esculetin (50 and 80 µM) did not significantly affect fibrinogen-induced aggregation of elastase-treated platelets; however, it markedly blocked integrin αIIbβ3 activation by interrupting the binding of fluorescein isothiocyanate-labeled PAC-1. In addition, neither ODQ nor SQ22536 significantly reversed esculetin-mediated antiplatelet activity stimulated by collagen. Nitroglycerin and prostaglandin E1 significantly increased VASP phosphorylation, but esculetin had no effect in this reaction, the values being almost identical with those of normal platelets. Furthermore, esculetin, at its maximum concentration of 80 µM significantly reduced the phosphorylation of IκBα and p65 and reversed IκBα degradation in collagen-induced platelets. These results suggest that the NF-κB-dependent αIIbβ3 inhibition of esculetin might represent a novel feedback inhibitory mechanism to regulate platelet functions.

Keywords
esculetin, coumarin, integrin αIIbβ3, VASP, cAMP/cGMP, NF-κB

Received: August 30th, 2019; Accepted: November 21st, 2019.

Platelet activation is induced by several compounds, such as adenosine diphosphate (ADP), thromboxane A2, thrombin, and collagen. Dense granules in the platelets release ADP, which plays a central role in aggregation by increasing platelet activation prompted by numerous factors. Platelet aggregation is divided into two phases namely inside-out and outside-in pathways. A major secondary messenger molecule, inositol triphosphate (IP3), together with diacylglycerol, formed during the inside-out pathway increase intracellular calcium concentration and stimulate the elevation of several enzymes such as protein kinase C (PKC). The termination of the first phase of platelet aggregation ends with fibrinogen receptor (αIIbβ3 integrin) activation and phosphorylation. In contrast, substances like nitric oxide (NO) may adversely control platelet activation. Guanylyl cyclase produced by NO increases cyclic 3′-5′guanosine monophosphate (cGMP), which stops platelet aggregation via either independent-on or dependent-on PKG

1Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan
2Division of Cardiology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan
3Translational Medicine Center, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan
4Department of Chemistry, North Eastern Hill University, Shillong, India
5Department of Pharmacology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan

*The authors Chih-Wei Hsia and Kou-Gi Shyu contributed equally to the work.

Corresponding Authors:
Chih-Hao Yang, Department of Pharmacology, School of Medicine, Taipei Medical University, 250 Wu-Hsing St, Taipei 110, Taiwan.
Email: chyang@tmu.edu.tw
Joen-Rong Sheu, Graduate Institute of Medical Sciences, Taipei Medical University, 250 Wu-Hsing St, Taipei 110, Taiwan.
Email: sheujr@tmu.edu.tw
Several proteins are established as PKG targets such as inositol triphosphate receptor, phosphodiesterase 5, and vasodilator-stimulated phosphoprotein (VASP). Besides, NO inhibits ADP-induced platelet adhesion in a cGMP-independent way by reducing αIIbβ3 integrin activation.

Moreover, endothelium-derived prostacyclin (PGI₂) released from the platelets binds to IP receptors which can induce the intracellular accumulation of cyclic 3′-5′ adenosine monophosphate (cAMP), which initiates AMP-dependent protein kinase (PKA) that phosphorylates VASP at serine residue 157. A study has shown that cAMP inhibits platelet aggregation via phosphorylation of VASP following integrin αIIbβ3 inactivation. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in platelets has been reported to act

Figure 1. Effect of esculetin on fibrinogen-induced elastase-treated platelet aggregation and αIIbβ3 integrin activation in washed human platelets. (a) Chemical structure of esculetin (C9H6O4). (b) The elastase-treated human platelets were preincubated with either esculetin (50 or 80 µM) or 0.1% dimethyl sulfoxide (DMSO) for 1 minute and then fibrinogen (200 µg/mL) was added to trigger platelet aggregation. (c) Washed platelets (3.6 × 10⁸ cells/mL) were preincubated with either (b) 0.1% DMSO or esculetin (c, 50 µM; d, 80 µM) and fluorescein isothiocyanate (FITC)-PAC-1 (2 µg/mL) for 3 minutes in either the (a) absence or (b–d) presence of collagen (1 µg/mL). ***P < 0.001, compared with the resting control; ###P < 0.001, compared with the 0.1% DMSO-treated group.
independently of gene regulation, and, after platelet activation, IκB is phosphorylated and degraded. Therefore, compounds that elevate cAMP production, increase VASP phosphorylation, and reduce NF-κB and integrin αIIbβ3 activation would be useful for the prevention of platelet-related cardiovascular diseases.

Esculetin, the main active component of Cortex Fraxini, has a similar nucleus to that of warfarin, a well-known antiplatelet drug. This coumarin-derived antioxidant has anti-inflammatory, anti-proliferative, and anti-tumor activities. Esculetin exerted an anti-lipidperoxidative effect in rats by dampening of isoproterenol-induced myocardial infarction and free radical scavenging properties. Our recent study showed that esculetin exerts antiplatelet effects by reducing phospholipase Cγ2 (PLCγ2) and the PKC cascade, hydroxyl radical formation, and Akt activation. In continuation of this study, here we aimed to investigate the role of NF-κB in the anti-aggregating effects of esculetin in human platelets activated with collagen. We also evaluated the downstream cyclic nucleotides signaling pathway, namely, VASP phosphorylation, and integrin αIIbβ3 activation in esculetin treated human washed platelets.

Integrin αIIbβ3 classically shows bidirectional signaling. Agonist stimulated signals result in an upsurge in the affinity of αIIbβ3 for extracellular ligands, which, in turn, activates outside-in signaling within the cell initiated by receptor ligation. An initial consequence of αIIbβ3 outside-in signaling is platelet spreading. The β3 subunit phosphorylated upon platelet activation via inside-out signaling and the binding affinity to fibrinogen of αIIbβ3 increased. The actions of outside-in signaling, including c-Src phosphorylation and activation, begin after β3 Tyr773 phosphorylation. In this study, the addition of fibrinogen caused aggregation of elastase-treated platelets and this aggregation was not inhibited by esculetin (50 and 80 µM, Figure 1(b)), indicating that it may not directly interrupt the association between fibrinogen and its receptors on platelet surfaces.

The adhesion of fibrinogen to trigger αIIbβ3 integrin is the last general pathway in platelet aggregation. Platelet filopodial extension, spreading, aggregation, and granule secretion are known to be regulated by αIIbβ3-induced signals. A study found that the agonists’ thrombin and collagen increased platelet αIIbβ3 activation when compared with non-activated platelets. These authors reported that pre-treatment with BAY 60-2770, a potent sGC activator, significantly increased collagen, induced αIIbβ3 activation. Here, to examine whether esculetin interrupts integrin αIIbβ3 activation, the binding of

---

**Figure 2.** The influence of cyclic nucleotides on esculetin-mediated platelet aggregation and vasodilator-stimulated phosphoprotein (VASP) phosphorylation. (a) Washed platelets (3.6 × 10^8 cells/mL) were incubated with 10 µM prostaglandin E1 (PGE1), 10 µM nitroglycerin (NTG), and 80 µM esculetin for 3 minutes in the presence of either SQ22536 (100 µM) or ODQ (10 µM), and then collagen (1 µg/mL) was added to trigger platelet aggregation. (b) Washed platelets were preincubated with 10 µM PGE1, 10 µM NTG, or esculetin (50 or 80 µM) for 3 minutes. **P<0.001, compared with the resting control.**
the fluorescein isothiocyanate (FITC)-conjugated PAC-1 mAb that reacts with the activation-induced conformational epitope of integrin $\alpha_{\text{IIb}}\beta_3$ was analyzed through flow cytometry (Figure 1 (c)). As anticipated, this (PAC-1) showed prominent integrin $\alpha_{\text{IIb}}\beta_3$ activation in the presence of collagen. On the inhibitory effect of esculetin, we found that at 50 and 80 $\mu$M, it significantly reduced integrin $\alpha_{\text{IIb}}\beta_3$ activity inhibited by collagen, showing that esculetin may influence the binding of PAC-1 to the activated integrin $\alpha_{\text{IIb}}\beta_3$.

Activation of platelets has been recognized to inhibit cAMP generation and cyclic nucleotide-dependent protein kinase activity. Hence, the effects of esculetin on platelet cAMP and cGMP levels were examined in this study. When nitroglycerin (NTG) and prostaglandin E$_1$ (PGE$_1$) were added to platelets before triggering of aggregation with collagen, they produced substantial inhibition of platelet aggregation, as shown in Figure 2(a). Moreover, ODQ (1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one, 10 $\mu$M), a guanylate cyclase inhibitor and SQ22536 (9-((tetrahydro-2-furanyl)-9H-purin-6-amine, 100 $\mu$M), an adenylyl cyclase inhibitor, considerably reversed the NTG and PGE$_1$-mediated inhibition of platelet aggregation stimulated by collagen. In addition, neither ODQ nor SQ22536 significantly reversed esculetin-mediated anti-platelet activity stimulated by collagen. VASP is a substrate of cAMP and cGMP-dependent protein kinases (PKA and PKG), and its stimulation by the increased activity of these kinases prevents platelet activation. As shown in Figure 2(b), PGE$_1$ and NTG could significantly increase VASP phosphorylation; however, esculetin did not increase VASP phosphorylation. This result suggested that esculetin inhibited platelet aggregation independently of cAMP/cGMP-mediated VASP phosphorylation.

Some part of the catalytic PKA molecules is bound to IKB in an NF-kB–IKB complex. Activation of cells with agonists of NF-kB activity separates NF-kB from IKB, resulting in IKB degradation and release, and cAMP-independent activation of PKA. The NF-kB complex plays a major role in the differentiation and maturation of megakaryocyte and is also expressed in platelets. Collagen activates the PI3K pathway that stimulates PKC and PKB that are elaborated in platelet activation. Conversely, both pathways excite IKK and degradation of an NF-kB–IKB–PKA complex, leading to free active PKA that phosphorylates VASP and other substrates involved in platelet inhibitory pathways. Here, we show that collagen significantly induced the phosphorylation of IKBz and p65 at 5 minutes (Figure 3 (a,b)) and also induced IKBz degradation after 10 minutes of stimulation (Figure 3 (c)). However, of the 50 and 80 $\mu$M pretreatment with esculetin, the maximal concentration of 80 $\mu$M significantly reduced the phosphorylation of IKBz (Figure 4 (a)) and p65 (Figure 4 (b)), and reversed IKBz degradation (Figure 4 (c)) in collagen-induced washed human platelets. These data showed that inhibition of NF-kB by esculetin may interfere with collagen-induced platelet activation and may be a potential target for the treatment of cardiovascular diseases.

In conclusion, the results show that platelet activation reduces the phosphorylation of VASP and activates NF-kB, subsequently inducing $\alpha_{\text{IIb}}\beta_3$ activation that is significantly involved in the platelet inhibitory pathways. Esculetin, a coumarin derivative, inhibited collagen stimulated $\alpha_{\text{IIb}}\beta_3$ integrin activation and NF-kB signaling events, independent of cAMP/cGMP. These results suggest that NF-kB-dependent $\alpha_{\text{IIb}}\beta_3$ inhibition of esculetin represents a novel feedback inhibitory mechanism to regulate platelet functions.

**Experimental**

**Materials**

Esculetin (Figure 1 (a)), elastase, collagen (type I), heparin, PGE$_1$, NTG, SQ22536, ODQ, and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA) and NF-kB from Cell Signaling (Beverly, MA, USA). A 0.1% dimethyl sulfoxide (DMSO) solution was used to dissolve esculetin.

**Preparation of Platelet Suspension and Platelet Aggregation**

The study procedure and concerned subjects were reviewed and approved by the Review Board, Taipei Medical University, Taipei, Taiwan (TMU-JIRB-N201612050). This study conformed to the directives of the Helsinki Declaration. All human volunteers associated with this study were advised to avoid taking any drugs that affect platelet aggregation for at least 14 days before the blood collection. All subjects provided written informed consent. The washed platelet suspensions were prepared as described in our previous study. Platelet aggregation was determined using a lumi-aggregometer (Payton Associates, Scarborough, ON, Canada), as previously described. The suspensions of platelets (3.6 × 10$^8$ cells/mL) were preincubated with either esculetin (50 and 80 $\mu$M) or an isovolumetric solvent control (0.1% DMSO) for 3 minutes before agonists were added. The reaction proceeded for 6 minutes, and the level of aggregation was calculated in light transmission units.

**Flow Cytometric Analysis of Integrin $\alpha_{\text{IIb}}\beta_3$ Activation**

Integrin $\alpha_{\text{IIb}}\beta_3$ activation was detected by flow cytometry by the method described previously in human platelets. Concisely, esculetin (50 and 80 $\mu$M) was added to the washed platelets (3.6 × 10$^8$ cells/mL) and subjected to FITC-conjugated PAC-1 mAb (2 $\mu$g/mL) for 3 minutes, followed by stimulation with collagen (1 $\mu$g/mL) for another 5 minutes. The total incubated suspensions were examined for fluorescein-labeled platelets using a flow cytometer (FACScan system; Becton Dickinson, San Jose, CA, USA).

**Immunoblotting**

Either esculetin (50 or 80 $\mu$M) or 0.1% DMSO was added to washed platelets for 3 minutes, and collagen (1 $\mu$g/mL) was
subsequently added to induce platelet activation. An equal amount of 80 µg total protein was loaded onto 12% SDS-PAGE, and proteins were separated and electrotransferred to polyvinylidene difluoride membranes using a semidy transfer unit (Bio-Rad, Hercules, CA, USA).

**Statistical Analysis**

The results are stated as means ± standard error of the means. The differences between the groups were assessed using analysis of variance (ANOVA). If a significant variation was found in the ANOVA results between the group means, the groups were normalized by using the Student-Newman-Keuls method. A P value of <0.05 designated statistical significance.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: We acknowledge the Ministry of Science and Technology of Taiwan (MOST 107-2320-B-038-035-MY2 and MOST 108-2320-B-038-031-MY3), Taipei Medical University (DP2-107-21121-N-02), and Shin Kong Wu Ho-Su Memorial Hospital-Taipei Medical University (SKH-TMU-104-05) for their financial support for this study.

**ORCID ID**

Chih-Wei Hsia https://orcid.org/0000-0002-4508-6181

**References**

1. Gachet C. ADP receptors of platelets and their inhibition. *Thromb Haemost*. 2001;86(1):222-232.
2. Stalker TJ, Newman DR, Ma P, et al. Platelet signaling. *Handb Exp Pharmacol*. 2012;210:59-85.
3. Fong KP, Zhu H, Span LM, et al. Directly activating the integrin αIIbβ3 initiates outside-in signaling by causing αIIbβ3 clustering. *J Biol Chem. 2016;291(22):11706-11716.*

4. Smolenski A. Novel roles of cAMP/cGMP-dependent signaling in platelets. *J Thromb Haemost.* 2012;10(2):167-176.

5. Oberprieler NG, Roberts W, Graham AM, Homer-Vanniassinkam S, Naseem KM. Inhibition of ADP-induced platelet adhesion to immobilised fibrinogen by nitric oxide: evidence for cGMP-independent mechanisms. *Biochem Pharmacol.* 2007;73(10):1593-1601.

6. Horstrup K, Jablonka B, Hönig-Liedl P, Just M, Kochsieck K, Walter U. Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition. *Eur J Biochem.* 1994;225(1):21-27.

7. Li Z, Ajdic J, Eigenthaler M, Du X. A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. *Blood.* 2003;101(11):4423-4429.

8. Liu F, Morris S, Epps J, Carroll R. Demonstration of an activation regulated NF-κB/IκBα complex in human platelets. *Thromb Res.* 2002;106(4-5):199-203.

9. Kwon OS, Choi JS, Islam MN, Kim YS, Kim HP. Inhibition of 5-lipoxygenase and skin inflammation by the aerial parts of *Artemisia capillaris* and its constituents. *Arch Pharm Res.* 2011;34(9):1561-1569.

10. Yun E-S, Park S-S, Shin H-C, et al. P38 MAPK activation is required for esculetin-induced inhibition of vascular smooth muscle cells proliferation. *Toxicol In Vitro.* 2011;25(7):1335-1342.
11. Park S-S, Park S-K, Lim J-H, et al. Esculetin inhibits cell proliferation through the Ras/ERK1/2 pathway in human colon cancer cells. *Oncol Rep*. 2011;25(1):223-230.

12. Karthika P, Rajadurai M, Ganapathy P, et al. Preventive effect of esculetin on lipid peroxides and antioxidants in isoproterenol-induced myocardial infarction in Wistar rats. *J Pharm Res*. 2012;5:915-918.

13. Hsia C-W, Lin K-C, Lee T-Y, et al. Esculetin, a coumarin derivative, prevents thrombosis: inhibitory signaling on PLCγ2-PKC-AKT activation in human platelets. *Int J Mol Sci*. 2019;20(11):E2731.

14. Varga-Szabo D, Pleines I, Nieswandt B. Cell adhesion mechanisms in platelets. *Arterioscler Thromb Vasc Biol*. 2008;28(3):403-412.

15. Senis YA, Mazharian A, Mori J. Src family kinases: at the forefront of platelet activation. *Blood*. 2014;124(13):2013-2024.

16. Payrastre B, Missy K, Trumel C, et al. The integrin αIIb/β3 in human platelet signal transduction. *Biochem Pharmacol*. 2000;60(8):1069-1074.

17. Sudo T, Ito H, Kimura Y. Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) by the anti-platelet drug, cilostazol, in platelets. *Platelets*. 2003;14(6):381-390.

18. Aszódi A, Pfeifer A, Ahmad M, et al. The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. *Embo J*. 1999;18(1):37-48.

19. Zhong H, SuYang H, Erzdjument-Bromage H, Tempst P, Ghosh S. The transcriptional activity of NF-κB is regulated by the IκB-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell*. 1997;89(3):413-424.

20. Kim KW, Kim SH, Lee EY, et al. Extracellular signal-regulated kinase/90-KDa ribosomal S6 kinase/nuclear factor-kappa B pathway mediates phorbol 12-myristate 13-acetate-induced megakaryocytic differentiation of K562 cells. *J Biol Chem*. 2001;276(16):13186-13191.

21. Gambaryan S, Kolsar A, Rukoyatkina N, et al. Thrombin and collagen induce a feedback inhibitory signaling pathway in platelets involving dissociation of the catalytic subunit of protein kinase A from an NFκB-IκB complex. *J Biol Chem*. 2010;285(24):18352-18363.

22. Sheu JR, Lee CR, Lin CH, et al. Mechanisms involved in the anti-platelet activity of *Staphylococcus aureus* lipoteichoic acid in human platelets. *Thromb Haemost*. 2000;83(5):777-784.

23. Hsia C-H, Velusamy M, Sheu J-R, et al. A novel ruthenium (II)-derived organometallic compound, TQ-6, potently inhibits platelet aggregation: *Ex vivo* and *in vivo* studies. *Sci Rep*. 2017;7(1):9556.