J Appl Biol Chem (2021) 64(3), 317–322
https://doi.org/10.3839/jabc.2021.043

Online ISSN 2234-7941
Print ISSN 1976-0442

Article: Bioactive Materials

Antiplatelet activity of esculetin through the down-regulation of PI3K/MAPK pathway

Dong-Ha Lee[1,2]

Received: 5 August 2021 / Accepted: 2 September 2021 / Published Online: 30 September 2021

Abstract Among the different cardiovascular disorders (CVDs), the activation of platelets is a necessary step. Based on this knowledge, therapeutic treatments for CVDs that target the disruption of platelet activation are proving to be worthwhile. One such substance, a bioactive 6,7-dihydroxy derived from coumarin, is 6,7-Dihydroxy-2H-1-benzopyran-2-one (esculetin). This compound has demonstrated several pharmacological effects on CVDS as well as various other disorders including diabetes, obesity, and renal failure. In various reports, esculetin and its effect has been explored in experimental mouse models, human platelet activation, esculetin-inhibited collagen, and washed human platelets exhibiting aggregation via arachidonic acid. Yet, esculetin affected aggregation with agonists like U46619 or thrombin in no way. This study investigated esculetin and how it affected human platelet aggregation activated through U46619. Ultimately, we confirmed that esculetin had an effect on the aggregation of human platelets when induced from U46619 and clarified the mechanism. Esculetin interacts with the downregulation of both phosphoinositide 3-kinase/Akt and mitogen-activated protein kinases, important phosphoproteins that are involved in activating platelets and their signaling process. The effects of esculetin reduced TXA_2 production, phospholipase A_2 activation, and platelet secretion of intracellular granules (ATP/serotonin), ultimately causing inhibition of overall platelet aggregation. These results clearly define the effect of esculetin in inhibiting platelet activity and thrombus formation in humans.

Dong-Ha Lee (✉)
E-mail: dhlee@nsu.ac.kr

[1]Department of Biomedical Laboratory Science, Namseoul University, Cheonan 31020, Republic of Korea

[2]Molecular Diagnostics Research Institute, Namseoul University, Cheonan 31020, Republic of Korea

Keywords Cytosolic phospholipases A2 · 6,7-Dihydroxy-2H-1-benzopyran-2-one · Mitogen-activated protein kinases · Phosphoinositide 3-kinase · Thromboxane A_2

Introduction

Human platelets are cells involved in the hemostasis of damaged blood vessels and are well known as cells involved in circulatory disorders including thrombosis, myocardial infarction, and atherosclerosis. Since platelets are cells that can directly affect cardiovascular disease, many drugs have been developed to inhibit them, and continuous research is being actively conducted. However, the mortality rate from cardiovascular disease has not decreased [1]. Therefore, it is urgent to identify an effective and stable antithrombotic component.

When platelets are activated, cytoplasmic membrane phospholipids are hydrolyzed and the released arachidonic acid is converted to TXA_2 and secreted [2,3]. The aggregation of platelets is amplified continuously when the agonist TXA_2 is released and binds to receptors of membranes found on other platelets [4]. Furthermore, an analog of TXA_2 known as U46619 (9,11-dideoxy 9a,11a-methanoepoxy prostaglandin F2a) raises levels of Ca^{2+} within the cytoplasm and further causes cytoskeletal proteins (myosin light chain and pleckstrin) to become phosphorylated, emerging as induced platelet aggregation [5,6].

An important group of proteins continuously being studied for their involvement during the process of intracellular signaling are mitogen-activated protein kinases (MAPK), and these kinases can further be ordered as p38, extracellular signal-regulated kinase (ERK), and N-terminal kinase (JNK) [7]. It is reported that JNK, ERK, and p38, are discovered in the platelets of humans and are activated through phosphorylation of various proteins when platelets are induced [8-10]. In particular, it has been reported that phosphorylation of MAPK in platelets is critically involved in the secretion of platelet granules [11,12]. In addition, MAPK strongly

phosphorylates the cytosolic phospholipases A_2 (PLA_2) in the cell membrane, initiating a reaction with phospholipids of the platelet membrane which generates and secretes arachidonic acid, and amplifies the activation and aggregation of peripheral platelets through conversion to TXA_2 [13,14]. In addition, phosphoinositide 3-kinase (PI3K) and Akt pathways in platelets play important roles in regulating platelet functions, including granule secretion and platelet aggregation [15].

The compound 6,7-Dihydroxy-2H-1-benzopyran-2-one (esculetin) is a component commonly found within roots of the genus *Artemisia* or *Scopolia*, and has demonstrated several pharmacological properties such as anticancer, anti-inflammatory, and antidiabetic [16]. Esculetin has been confirmed to provide a protective effect on the cardiovascular system. When esculetin was used as an oral pretreatment in rats with myocardial infarction induced by isoproterenol, an anti-lipperoxidation effect was shown, which is expected because of the free radical scavenging properties of esculetin [17]. Another study reported that esculetin regulates platelet activation by regulating NF-κB signaling in a cyclic nucleotide-independent manner [18]. In addition, according to a certain study, the effect of esculetin was confirmed in an experimental mouse model and human platelet activation, and it was confirmed that esculetin inhibited the aggregation of platelets which were induced by arachidonic acid and collagen [19]. However, the effect of esculetin was not shown in the results induced with U46619 or thrombin [19]. We tried to clarify the mechanism of action for esculetin by confirming the effect of esculetin on the PI3K/MAPK pathway.

Materials and Methods

Materials

Avention Corporation (Seoul, Korea) provided esculetin (Fig. 1). Chrono-Log Corporation (Havertown, PA, USA) provided the U46619. Cayman Chemical (Ann Arbor, MI, USA) provided assay kits (lactate dehydrogenase cytotoxicity, ATP), Thromboxane B2 kit (TXB_2), and EIA kit (serotonin). Cell Signaling (Beverly, MA, USA) provided lysis buffer as well as antibodies for western blotting. GE Healthcare (Buckinghamshire, UK) provided the PVDF (polyvinylidene difluoride) membrane and solution of ECL (enhanced chemiluminescence). Sigma Aldrich (Saint Louis, MO, USA) provided the other reagents.

Washed platelets (human)

The Korean Red Cross Blood Center (Suwon, Korea) produced the solution of acid-citrate-dextrose (2.45% glucose, 2.2% sodium citrate, 0.8% citric acid) with anticoagulated human platelet-rich plasma (PRP). PRP centrifuging took place for 10 min (125 g at 1,300× g) to remove any trace amounts of red blood cells, then a wash (2×) with washing buffer. Followed by reconstitution of platelets (washed) with suspension buffer ending in an ultimate concentration of 10^8 cells/mL. The temperature was maintained at 25 °C for all procedures to hold off platelet aggregation (due to low temperatures). The Institutional Review Board (Namseoul University, Cheonan, korea) (1041479-HR-201803-003) gave full approval for this experiment and all procedures.

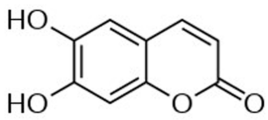

Fig. 1 Esculetin and its chemical structure. PIN: 6,7-Dihydroxy-2H-1-benzopyran-2-one, Chemical formula: C9H6O4, Molar mass: 178.14 g/moL

Platelet aggregation

Esculetin was added to dimethyl sulfoxide (DMSO) to a 0.1% concentration. The platelets (10^8 cells/mL), which had been washed, were pretreated (37 °C, 3 min) by adding various concentrations of esculetin, and then aggregation was induced with 0.5 μM U46619 and measured (5 min). Using an aggregometer, the aggregation of platelets was measured (1,000 rpm) (Chrono-Log, Havertown, PA, USA), and the aggregation capacity was calculated as an increased degree of light transmittance. A suspension buffer with a reference value of 0% permeability was used.

Cytotoxicity

Platelets (10^8 cells/mL), which had been washed, were pretreated (37 °C, 5 min) by adding varying concentrations of esculetin, and to remove debris from the cells, centrifuged (12,000× g, 15 min). To remove the supernatant, the lactate dehydrogenase (LDH) cytotoxicity assay kit (Cayman Chemical) was used, and the supernatant was then measured. The value of completely lysing platelets with 0.1% Triton X 100 was set to 100% to represent the positive control, and the esculetin value was presented as a percentage.

TXA_2 production

Washed platelets (10^8 cells/mL) were pretreated with esculetin of different concentrations (37 °C for 3 min), followed by agglutination reaction with 0.5 μM U46619 (5 min). After that, TXA_2 synthesis was stopped by treatment with 400 μL of 5 mM EDTA (ice-cold) along with 0.2 mM indomethacin. Thereafter, TXB_2 (TXA_2 metabolite) was analyzed with the TXB_2 ELISA kit (Cayman Chemical).

ATP and serotonin release measurement

Platelets (10^8 cells/mL), which had been washed, were pretreated with esculetin of different concentrations (37 °C, 3 min), followed by agglutination reaction with 0.5 μM U46619 (5 min). With an addition of 2 mM EDTA to stop the reaction, and then measurement of the released molecules (ATP or serotonin), found at the top

layer from centrifuging, with an ELISA-reader (Molecular Devices, San Jose, CA, USA) using ATP or serotonin kit.

Western blotting

Platelets (10^8 cells/mL), which were washed, were pretreated with esculetin of different concentrations (37 °C, 3 min), followed by agglutination reaction with 0.5 μM U46619 (5 min). Interruption of the reaction happened with the inclusion of the same amount of lysis buffer. Platelet lysate was analyzed using 8% SDS-PAGE after protein quantification with the use of a protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

Statistical analysis

Any measured results from the experiment were treated using mean ± SD and analyzed by ANOVA (analysis of variance). If a significant difference among the mean groups existed, it was analyzed with the Newman-Keuls method and marked between each group. When p <0.05, it was judged to have a significant meaning.

Results and Discussion

Effect of esculetin on U46619-induced platelet aggregation and Cytotoxicity

In this study, the effect of esculetin on U46619-induced human platelets and its inhibitory mechanism was identified. The maximum aggregation induction concentration of U46619, a TXA_2 analog that induces platelet aggregation, was found to be 0.5 μM, and 0.5 μM U46619 was used to induce aggregation in this experiment (Fig. 2A). When U46619-induced human platelets were treated with esculetin (50, 100, 300, 500 μM), a concentration-dependent inhibition pattern was confirmed (Fig. 2B). The half-maximal inhibitory concentration of esculetin was 151.9 μM (Fig. 2C). To evaluate any cytotoxicity among the platelets due to esculetin, LDH leakage was performed. Human platelets were treated with esculetin (50, 100, 300, 500 μM) to analyze LDH leakage, and the results showed no significance (Fig. 2D). This result makes it clear that esculetin has an inhibitory effect on

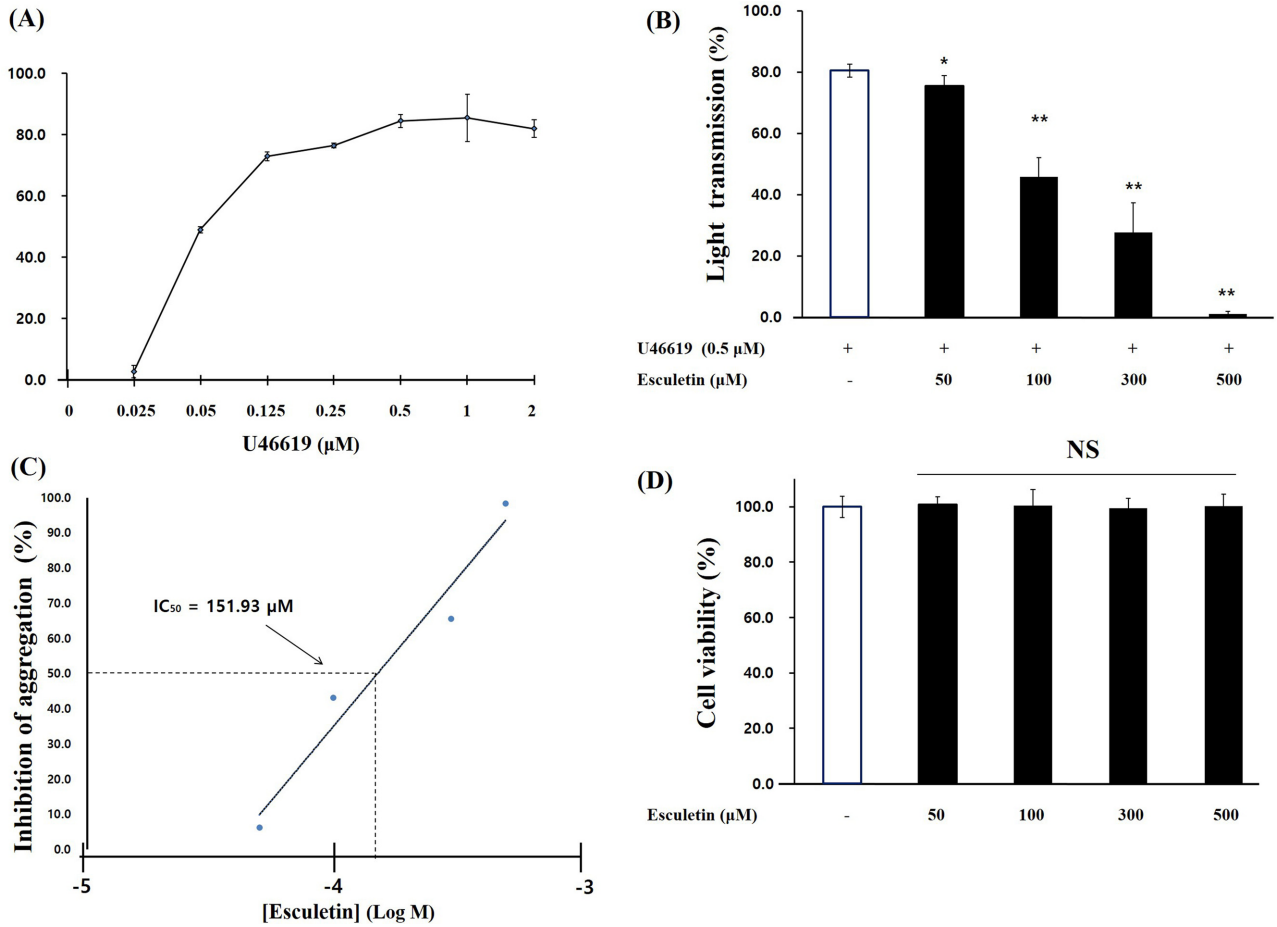

Fig. 2 Esculetin affecting cytotoxicity and aggregation of platelets. (A) The threshold of U46619 concentration on the aggregation of human platelets. (B) Esculetin affecting aggregation with U46619-induced human platelets. (C) The value for half-maximal inhibitory concentration (IC_{50}) of esculetin among U46619-induced human platelet aggregation. (D) Cytotoxicity and the effect of esculetin. Cytotoxicity and the aggregation of platelets were done as stated in the section, "Materials and Methods". The mean ± standard deviation (n =4) is used to express the data. Versus U46619-stimulated human platelets: *p <0.05, **p <0.001. Not significant, NS

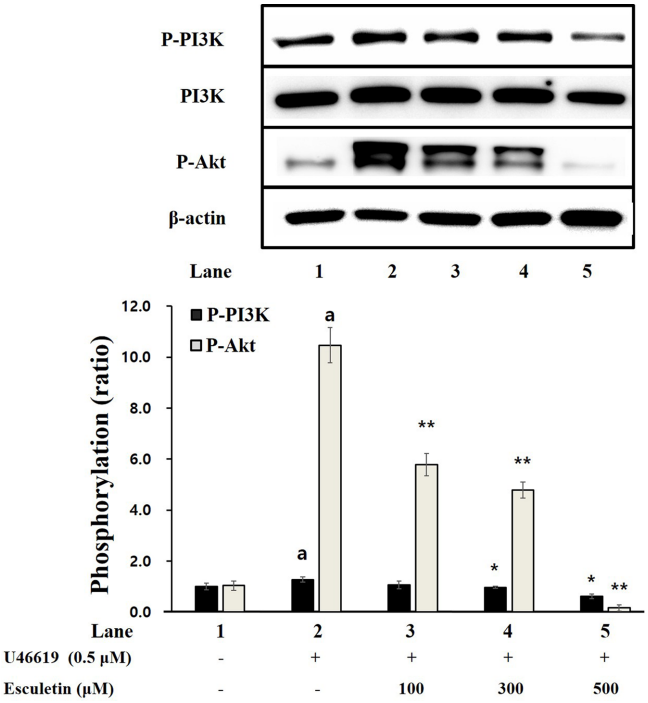

Fig. 3 Esculetin affecting PI3K/Akt phosphorylation. Western blot was done as stated in the section, "Materials and Methods". The mean ± standard deviation (n =4) is used to express the data. Versus non-stimulated platelets: ^{a}p <0.05; versus U46619-stimulated human platelets: *p <0.05, **p <0.001

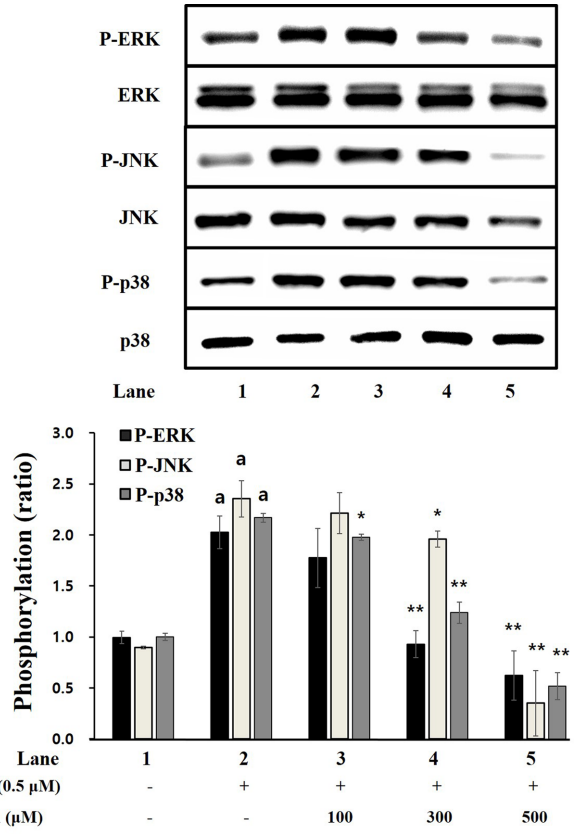

Fig. 4 Esculetin affecting MAPK phosphorylation. Western blot was done as stated in the section, "Materials and Methods". The mean ± standard deviation (n =4) is used to express the data. Versus non-stimulated platelets: ^{a}p <0.05; versus U46619-stimulated human platelets: *p <0.05, **p <0.001

U46619-induced platelets, unlike previous studies.

Effect of esculetin on PI3K/Akt and MAPK pathway

PI3K/Akt (phosphoproteins) participate in the release of platelet granules, and it was confirmed that esculetin did exhibit an effect in regards to PI3K/Akt being phosphorylated. As demonstrated in Fig. 3, the use of U46619 created an increase in the phosphorylation of PI3k/Akt that was considered significant in comparison to the intact cells, but the addition of esculetin significantly reduced the U46619-increased phosphorylation, thus revealing that esculetin inhibits PI3k/Akt U46619-induced phosphorylation (Fig. 3). Furthermore, the effects of esculetin were identified in regards to MAPK being phosphorylated and its relation to platelet granule release as well as TXA2 production. As depicted in Fig. 4, U46619 potently increased the amount of phosphorylated ERK/ JNK/ p38 (MAPKs) in comparison with intact cells. Moreover, the U46619-induced increase of phosphorylated MAPKs was significantly inhibited by esculetin (Fig. 4), demonstrating that esculetin controls the process of signaling for the aggregation of platelets through inhibiting MAPKs being phosphorylated.

PI3K/Akt pathway acts during intracellular signaling when platelets are activated, and their phosphorylation plays some major roles among platelet regulation and function, including the aggregation of platelets and the secretion of dense granules [16]. In addition, MAPK, a group of phosphorylating enzymes including ERK, c-Jun JNK, and p38 MAPK, are recognized as molecules that partake in platelet activation and aggregation [8]. Human platelets contain MAPK and reports show its activation via phosphorylation after platelets are activated by several agents [9-11].

Effect of esculetin on PLA_2 activation and TXA_2 production

According to a study by Mei-Chi et al., it was reported that phosphorylated MAPK (p38) is involved in platelet aggregation and is important for arachidonic acid release (TXA_2 precursor) and the production of TXA_2. When evaluating a component or substance for platelet inhibitory activity, it is TXA_2 production that is viewed as an important indicator due to TXA_2 performing like a potent autacoid causing activation and aggregation among platelets [20]. Accordingly, substances inhibiting TXA_2 production, such as aspirin and ozagrel, have been effectively applied as antiplatelet agents and are well known [21,22]. MAPK strongly phosphorylates the PLA_2 in the cell membrane to generate and secrete arachidonic acid from phospholipids in the platelet membrane and amplifies the activation and aggregation of peripheral platelets through conversion to TXA_2 [14,15].

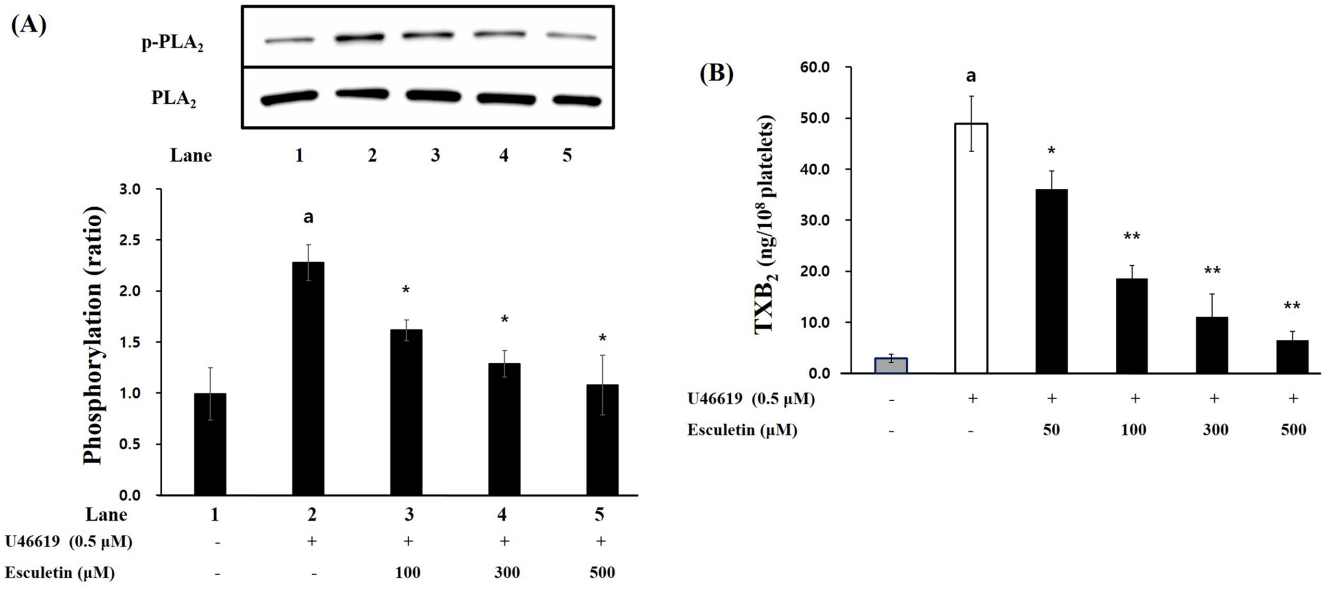

Fig. 5 Esculetin affecting TXA_2 generation and PLA_2 phosphorylation**.** (A) Esculetin and its effect on U46619-induced phosphorylation of PLA_2. (B) Effect of esculetin on U46619-induced TXA_2 generation. TXA_2 generation measurement and Western blot were done as stated in the section, "Materials and Methods". The mean ± standard deviation (n =4) is used to express the data. Versus non-stimulated platelets: ^{a}p <0.05; versus U46619-stimulated human platelets: *p <0.05, **p <0.001

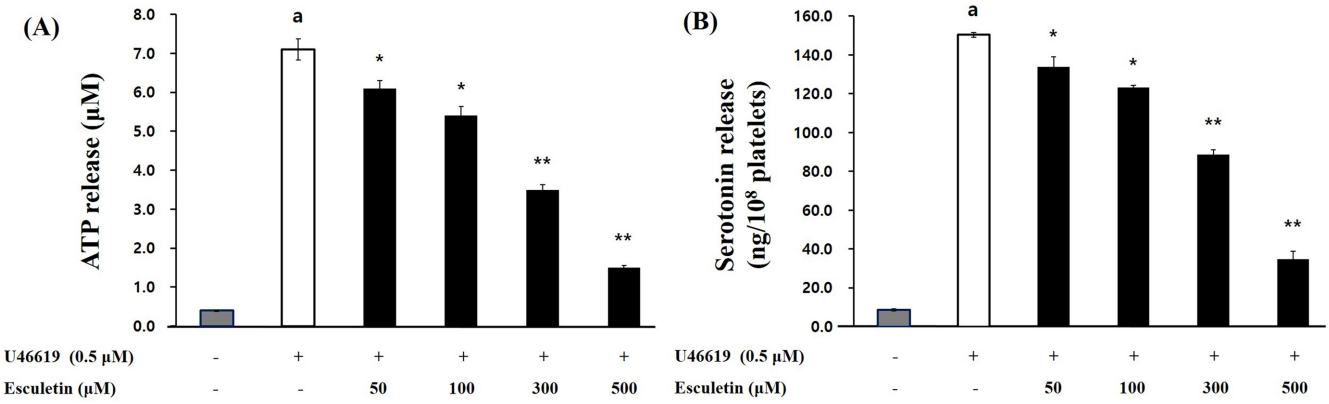

Fig. 6 Esculetin affecting granule secretion. (A) Esculetin and its effect on the release of ATP. (B) Esculetin and its effect on the release of serotonin. Measurement of granule secretion happened as stated in the section, "Materials and Methods". The mean ± standard deviation (n =4) is used to express the data. Versus non-stimulated platelets: ^{a}p <0.05; versus U46619-stimulated human platelets: *p <0.05, **p <0.001

As depicted in Fig. 5A, esculetin inhibited the phosphorylation of PLA_2 increased by U46619 in a concentration-dependent manner. In addition, the effect of esculetin on the production of TXA_2, an autacoid that amplifies platelet aggregation, was confirmed. As shown in Fig. 5B, TXA_2 production, which was 3.0±0.8 ng/10^8 cells in intact cells, was increased to 48.9±5.4 ng/10^8 cells by U46619. However, esculetin (50, 100, 300, and 500 μM) showed to significantly reduce the TXA_2 production to 36.1±3.6, 18.6±2.5, 11.0±4.5 and 6.5±1.7 ng/10^8 cells, respectively (Fig. 5B). This is thought to be the result of MAPK activity being inhibited by esculetin, further reducing the phosphorylation of PLA_2.

Effect of esculetin on granule secretion

The effect of esculetin was confirmed in relation to ATP/serotonin release in platelet aggregation, an index of granule release. U46619 (0.5 μM) caused the releasing of ATP to increase from 0.36±0.02 μM (intact cells) to 7.14±0.27 μM (Fig. 6A); however, the increased ATP release was significantly inhibited by esculetin (50, 100, 300 and 500 μM). In addition, U46619 (0.5 μM) increased serotonin release from 8.7±0.6 ng/10^8 cells in intact cells to 150.4±1.3 ng/10^8 cells. Yet, esculetin (50, 100, 300, and 500 μM) reduced the serotonin release increased by U46619 to 133.9±5.2, 123.3±1.1, 88.8±2.3 and 34.9±3.9 ng/10^8 cells, respectively (Fig. 6B). These results show that esculetin significantly

inhibits granule secretion.

It is acknowledged that granule secretion is vital in the formation of a thrombus due to its action of promoting platelet activation and recruiting circulating platelets to damaged blood vessels [23]. In this study, esculetin is considered to affect platelet activation and inhibition of thrombus formation by reducing ATP release and serotonin release from U46619-induced dense granules of platelets. These findings indicate that esculetin is an effective substance when it comes to inhibiting platelet activity and thrombus formation in humans.

Acknowledgments Funding for this paper was provided by Namseoul University

Conflict of interest The authors declare no conflict of interest.

References

1. 1Jackson SP (2011) Arterial thrombosis?insidious, unpredictable and deadly. Nat Med 17: 1423–1436. doi: 10.1038/nm.2515
2. Morello F, Perino A, Hirsch E (2009) Phosphoinositide 3-kinase esculetin in the vascular system. Cardiovasc Res 82: 261–271. doi: 10.1093/cvr/cvn325
3. Jennings LK (2009) Role of platelets in atherothrombosis. Am J Cardiol 103:4A–10A. doi: 10.1016/j.amjcard.2008.11.017
4. Sabatine MS, Jang IK (2000) The use of glycoprotein IIb/ IIIa inhibitors in patients with coronary artery disease. Am J Med 109: 224–237. doi: 10.1016/s0002-9343(00)00474-5
5. Cattanco M, Tenconi PM, Lecchi A, Mannucci PM (1991) In vitro effects of picotamide on human platelet aggregation, the release reaction and thromboxane B2 production. Thromb Res 62: 717–724. doi: 10.1016/0049-3848(91)90375-7
6. Su CY, Shiao MS, Wang CT (1999) Differential effects of ganodermic acid S on the thromboxane A2-signaling pathways in human platelets. Biochem Pharmacol 58: 587–595. doi: 10.1016/s0006-2952(99)00136-7
7. Adam F, Kauskot A, Nurden P, Sulpice E, Hoylaerts MF, Davis RJ (2010) Platelet JNK1 is involved in secretion and thrombus formation. Blood 115: 4083–4092. doi: 10.1182/blood-2009-07-233932
8. Bugaud F, Nadal-Wollbold F, Lévy-Toledano S, Rosa JP, Bryckaert M (1999) Regulation of c-jun-NH2 terminal kinase and extracellular-signal regulated kinase in human platelets. Blood 94:3800–3805
9. Kramer RM, Roberts EF, Strifler BA, Johnstone EM (1995) Thrombin induces activation of p38 MAP kinase in human platelets. J Biol Chem. 270: 27395–27398. doi: 10.1074/jbc.270.46.27395
10. Nadal-Wollbold F, Pawlowski M, Lévy-Toledano S, Berrou E, Rosa JP, Bryckaert M (2002) Platelet ERK2 activation by thrombin is dependent on calcium and conventional protein kinases C but not Raf-1 or B-Raf. FEBS Lett 531: 475–482. doi: 10.1016/s0014-5793(02)03587-1
11. Patrono C (1994) Aspirin as an antiplatelet drug. N Engl J Med 330: 1287–1294. doi: 10.1056/NEJM199405053301808
12. Flevaris P, Li Z, Zhang G, Zheng Y, Liu J, Du X (2009) Two distinct roles of mitogen-activated protein kinases in platelets and a novel Rac1-MAPK-dependent integrin outside-in retractile signaling pathway. Blood 113: 893–901. doi: 10.1182/blood-2008-05-155978
13. Kramer RM, Roberts EF, Um SL, Börsch-Haubold AG, Watson SP, Fisher MJ (1996) p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets. Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2. J Biol Chem 271: 27723–27729. doi: 10.1074/jbc.271.44.27723.
14. McNicol A, Shibou TS (1998) Translocation and phosphorylation of cytosolic phospholipase A2 in activated platelets. Thromb Res 92: 19–26. doi: 10.1016/s0049-3848(98)00097-8
15. Chuang WY, Kung PH, Kuo CY, Wu CC (2013) Sulforaphane prevents human platelet aggregation through inhibiting the phosphatidylinositol 3-kinase/Akt pathway. Thromb Haemost 109: 1120–1130. doi: 10.1160/TH12-09-0636
16. Karthika P, Rajadurai M, Ganapathy P, Kanchana G (2012) Preventive effect of esculetin on lipid peroxides and antioxidants in isoproterenol-induced myocardial infarction in Wistar rats. J Pharm Res 5: 915–918
17. Liang C, Ju W, Pei S, Tang Y, Xiao Y (2017) Pharmacological activities and synthesis of esculetin and its derivatives: A mini-review. Molecules 22: 387. doi: 10.3390/molecules22030387
18. Hsia CW, Shyu KG, Jayakumar T, Hsia CH, Velusamy M, Yang CH, Sheu JR (2019) Natural coumarin derivative esculetin regulates platelet activation via modulating NF-κB signaling in cyclic nucleotide-independent manner. Natural Product Communications 14(12): 1934578X19896663. doi: 10.1177/1934578X19896663
19. Hsia CW, Lin KC, Lee TY, Hsia CH, Chou DS, Jayakumar T, Velusamy M, Chang CC, Sheu JR (2019) Esculetin, a Coumarin Derivative, Prevents Thrombosis: Inhibitory Signaling on PLCγ2–PKC–AKT Activation in Human Platelets. Int J Mol Sci 20(11): 2731. doi: 10.3390/ijms20112731
20. Chang MC, Wang TM, Yeung SY, Jeng PY, Liao CH, Lin TY, Lin CC, Lin BR, Jeng JH (2011) Antiplatelet effect by p-cresol, a uremic and environmental toxicant, is related to inhibition of reactive oxygen species, ERK/p38 signaling and thromboxane A2 production. Atherosclerosis 219: 559–565. doi: 10.1016/j.atherosclerosis.2011.09.031
21. F Cipollone F, Patrignani P, Greco A, Panara MR, Padovano R, Cuccurullo F, Patrono C, Rebuzzi AG, Liuzzo G, Quaranta G, Maseri A. (1997) Differential suppression of thromboxane biosynthesis by indobufen and aspirin in patients with unstable angina. Circulation 96: 1109–1116. doi: 10.1161/01.cir.96.4.1109
22. Patrono C (2001) Aspirin: new cardiovascular uses for an old drug. Am J Med 110: 62S–65S. doi: 10.1016/s0002-9343(00)00645-8
23. Calderwood DA (2004) Integrin activation. J Cell Sci 117: 657–666. doi: 10.1242/jcs.01014