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Inhibitory effects of artemether on collagen-induced platelet aggregation via regulation of phosphoprotein inducing PI3K/Akt and MAPK

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Abstract Pathophysiological reaction of platelets in the blood vessel is an indispensable part of thrombosis and cardiovascular disease, which is the most common cause of death in the world. In this study, we performed in vitro assays to evaluate antiplatelet activity of artemether in human platelets and attempted to identify the mechanism responsible for protein phosphorylation. Artemether is a derivative of artemisinin, known as an active ingredient of Artemisia annua, which has been reported to be effective in treating malaria, and is known to function through antioxidant and metabolic enzyme inhibition. However, the role of artemether in platelet activation and aggregation and the mechanism of action of artemether in collagen-induced human platelets are not known until now. In this study, the effect of artesunate on collagen-induced human platelet aggregation was confirmed and the mechanism of action of artemether was clarified. Artemether inhibited the phosphorylation of PI3K/Akt and Mitogen-activated protein kinases, which are phosphoproteins that are known to act in the signal transduction process when platelets are activated. In addition, artemether decreased TXA_2 production and decreased granule secretion in platelets such as ATP and serotonin release. As a result, artemether strongly inhibited platelet aggregation induced by collagen, a strong aggregation inducer secreted from vascular endothelial cells, with an IC_{50} of 157.92 μM. These results suggest that artemether has value as an effective antithrombotic agent for inhibiting the activation and aggregation of human platelets through vascular injury.

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Keywords Artemether · Granule secretion · Mitogen-activated protein kinases · Platelet aggregation · PI3K/Akt · TXA_2

Introduction

Thrombosis and cardiovascular disease are global health issues and are due to abnormal changes of thrombocytes (platelets) found within blood vessels. A dietary or pharmacological solution is needed in order to combat the occurrence of CVD. An area that has shown promise and helped reduce CVD risk is the use of medicinal plants and their phytochemicals. As a result, pharmaceuticals used in oriental medicine to prevent and treat cardiovascular diseases are being studied globally [1]. The onset of inflammation begins the activation and aggregation of platelets by creating arachidonic acid (AA) from fatty acids. Following this step, active eicosanoids (eoxins, lipoxin, prostaglandins, and thromboxanes) are created through the breakdown of arachidonic acid via the cyclooxygenase enzyme system. Within cells of mammals, COX-1 and COX-2 activity exist and platelet cells are found expressing COX-1 [2]. The creation of prostaglandin (PG) G_2/PGH_2 from AA is a two-step process promoted through COX-1. Then PGH_2 is further changed into thromboxane A_2 (TXA_2) through the synthase known as thromboxane A. Thromboxane A_2 amplifies signals for strong agonists that can directly stimulate platelets like epinephrine, adenosine diphosphate, thrombin, and collagen [3].

Phosphoinositide 3-kinase (PI3K) is activated when stimulated agonists cause surface receptors to attach to PI3K and perform like second messengers prompting the activation of Akt found within the lining of platelets [3]. The loop that activates phosphorylation for Akt can be found at Thr308 and the hydrophobic motif is Ser473. Altering these regions brings maximum activation of Akt [4]. Using the signal transduction pathway, PI3K/Akt, the mammalian target of rapamycin (mTOR)

becomes phosphorylated at Ser2448 playing a major part in the aggregation of platelets [5]. The three subtypes of mitogen-activated protein kinases (MAPKs) located within in platelets are c-Jun N-terminal kinases (JNKs), p38, and extracellular signal-related kinases (ERKs). Through the use of platelets agonists, quick activation of MAPK has been demonstrated and this is important for a number of functions such as integrin signaling, granule secretion/spreading and the contraction of a thrombus [6,7]. Akt (protein kinase B), a protein kinase labeled as serine/threonine-specific, also aids in the function of aggregation (platelets), signaling integrins, secretion of granules, and thrombin contraction [4,8]. Given this information, the pathways of PI3K, Akt, MAPK, and mTOR are all crucial for the proper functioning of platelet aggregation and changes in their function cause the activation of platelet integrin known as $\alpha IIb\beta_3$ and result in the impairment of forming a thrombus, thus making the pathways interesting targets for therapeutic methods [6,7,9].

Artemether is a methyl ether derivative of artemisinin, which is a peroxide-containing lactone isolated from the antimalarial plant Artemisia annua. It is a relatively lipophilic component, which can be obtained by processing Artemisinin, a component of the natural plant *Artemisia annua*. Evidence shows artemether being effective in the treatment of malaria due to the generation of cytotoxic radicals through the interaction of iron ions or ferriprotoporphyrin IX (heme) in vacuoles of parasites [10]. A suggested mechanism of artemether is the exertion of both metabolic and oxidative stress on cells via inhibition of metabolic enzymes and antioxidants, ultimately causing a reduction in parasite growth and lesion [11]. At this time, artemether and its relationship to activation and also aggregation of platelets, as well as a mechanism by which artemether acts on human platelets induced by collagen, still remains undetermined. Accordingly, the reason for conducting this study was to bring light to artemether and its antiplatelet ability through the examination of how artemether interacts with and affects the process of platelets activated and induced by collagen.

Materials and Methods

Materials

Avention in Siheung (Korea) supplied artemether (Fig. 1). Chrono-Log (Havertown, PA, USA), supplied collagen. The assay kit for serotonin manufactured by Labor Dignostika Nord GmbH (LDN) in Nordhorn (Niedersachsen, Germany). Assay kits for TXB_2 and ATP were manufactured in Ann Arbor, Michigan (USA) by Cayman Chemical. Inhibitor cocktails (protease and phosphatase) manufactured by GenDEPOT in Barker, Texas (USA). The following antibodies were supplied by Cell Signaling Corporation from Beverly, Massachusetts (USA): Akt, phospho-Akt (Ser473/Thr308), PI3K, phospho-PI3K, ERK1/2, Phospho-ERK1/2 (Thr202/Tyr204), p38, phospho-p38, β-actin, JNK, phospho-JNK. The bicinchoninic (BCA) Protein Assay Kit (Bicinchoninic acid Pierce™) was provided by Thermo Fischer Scientific in Waltham, Massachusetts (USA) and the membrane (polyvinylidene fluoride) provided by Pall Life Sciences in Port Washington, New York (USA).

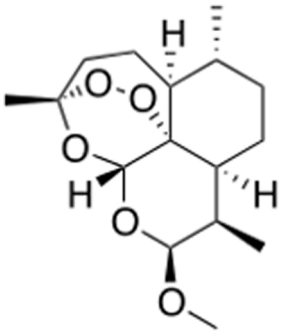

Fig. 1 The structure of artemether. PIN: Dihydroartemisinin methyl ether, Chemical formula: $C_{16}H_{26}O_5$, Molar mass: 298.379 g/mole

Washed platelets and preparation

Human platelet-rich plasma containing CPDA solution supplied by Korean Red Cross Blood Center in Suwon (Korea). The plasma was centrifuged (10 min) at 125 g allowing for the removal of red blood cells, then further centrifuged (10 min) at 1300 g to develop a pellet of platelets. Using wash buffer with pH 6.5 (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) wash the pellet of platelets twice. Resuspend using a suspension buffer with pH 6.9 (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) until the concentration reads 5×10^8/mL. These methods were done at a temperature of 25 °C so platelet activation due to low temperature could be avoided. Namseoul University approved this study via approval from their Bioethics Institution Review Board (1041479-HR-201803-003).

Platelet aggregation response and measurement

The light-transmission aggregation assay from Chrono-Log in Havertown, Pennsylvania (USA) allowed for proper platelet aggregation assessment. Performed in a temperature of 37 °C and keeping a stir speed of 1000 rpm. Human platelets that were washed were then pre-incubated with artemether of varying concentrations and $CaCl_2$ (2 mM) for a length of 2 min, then stimulation through collagen, and monitored for 5 min. The degree (percentage %) to which platelet aggregation occurred was determined upon completion of a 5 min reaction time.

Measurement of cytotoxicity by lactate dehydrogenase

Lactate dehydrogenase (LDH) that leaked from the cytoplasm was used to calculate the amount of platelet cytotoxicity. After washing human platelets (10^8/mL), the varying concentrations of

artemether were added at a temperature of 37 °C and for a length of 30 min and finally centrifuged (5 min) at 2000 g in a room temperature (RT) environment. The assay kit for cytotoxicity provided by Promega in Madison, Wisconsin (USA) and the SpectraMax M2e from Molecular Devices in Sunnyvale, California (USA) measured the supernatant. For cytotoxicity, the maximum value was recorded at 0.05% triton X-100.

Measurement of TXB_2

To prevent AA from being metabolized to TXA_2, ice-cold 5 mM EDTA (0.2 mM indomethacin) was mixed to terminate platelet aggregation, then platelet aggregate supernatant was centrifuged (5 min) at 2000 g at 4 °C to develop a platelet aggregate mixture. The quantity of TXB_2 (TXA_2 stable metabolite) was processed with the TXB_2 EIA kit from Cayman Chemical and the SpectraMax M2e manufactured by Molecular Devices in Sunnyvale, California (USA).

Assay for release of ATP and serotonin

The supernatant consisting of aggregated platelets was separated from mixture using centrifugation (5 min) at 2000 g at a temperature of 4 °C. To determine the release of ATP and serotonin, the ATP assay manufactured by Cayman Chemical and serotonin ELISA manufactured by LDN in Nordhorn (Germany) were used with the SpectraMax M2e from Molecular Devices in Sunnyvale, California (USA).

Immunoblotting analysis and protein extraction

Once aggregation of platelets occurred, the lysing of samples was carried out using lysis buffer, RIPA (50 mM pH 7.5 Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 2 mM EDTA), and phosphatase and protease inhibitors. Lysate decontamination happened at 5000 g and a duration of 10 min and a temperature of 4 °C. Concentration of protein measured with BCA protein acid assay kit. Protein obtained via platelet lysates then separated in equal amounts by SDS-polyacrylamide gel electrophoresis, moved to membranes (PVDF), blocked (BSA 2%) in buffer (TBS), and finally introduced to primary antibody at a temperature of 4 °C (overnight), then placed in room temperature (1 h). Visualization of protein bands was achieved with chemiluminescent substrate and the Luminescent Image Analyzer LAS-4000 from Fujifilm in Tokyo (Japan) for photographing. The Scion Image from Scion in Frederick, Maryland (USA) provided densitometry analysis on data acquired from three independent experiments.

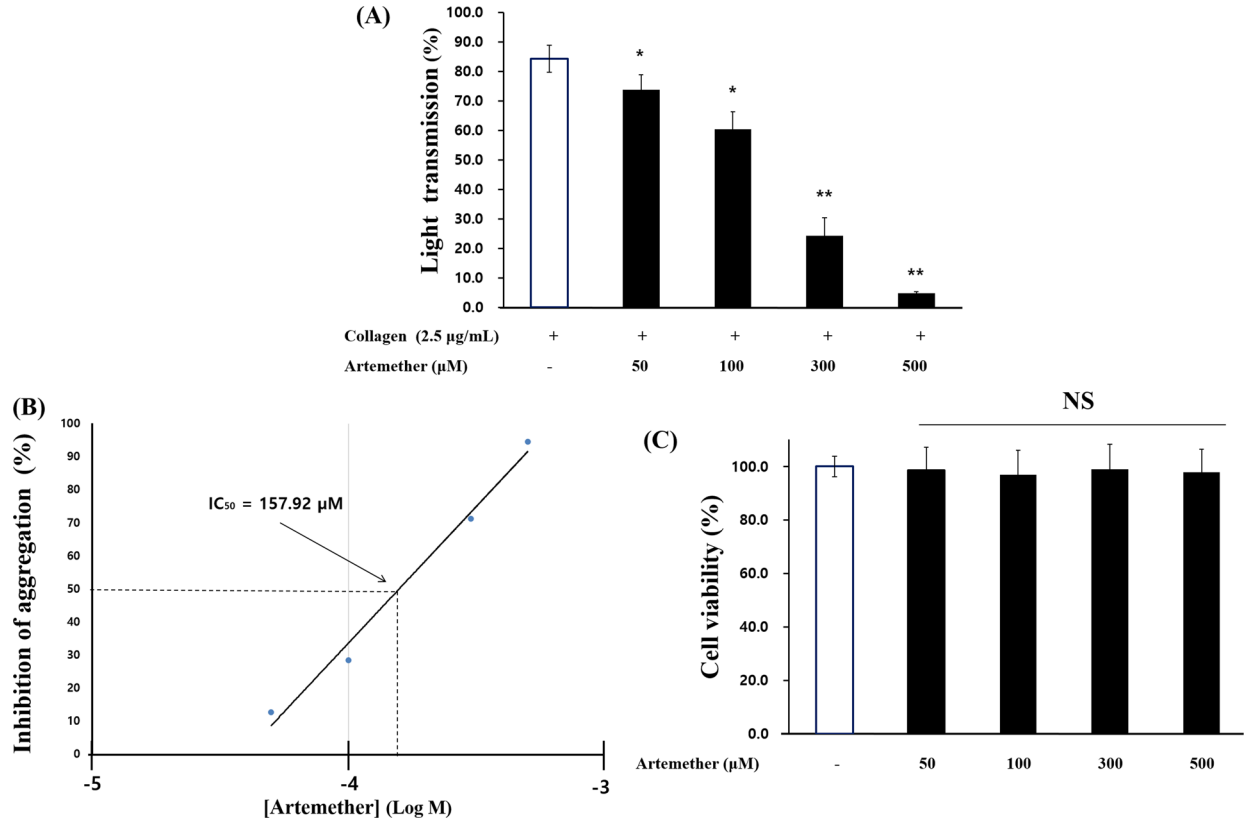

Fig. 2 Effects of artemether on collagen-induced platelet aggregation. (A) The effect of artemether pretreatment on collagen-stimulated platelet aggregation. (B) IC_{50} value of artemether on collagen-induced platelet aggregation. (C) Cytotoxicity of artemether on human platelets. Data are expressed as mean ± SD (n =4). *p <0.05, **p <0.001 compared with the collagen-stimulated platelets

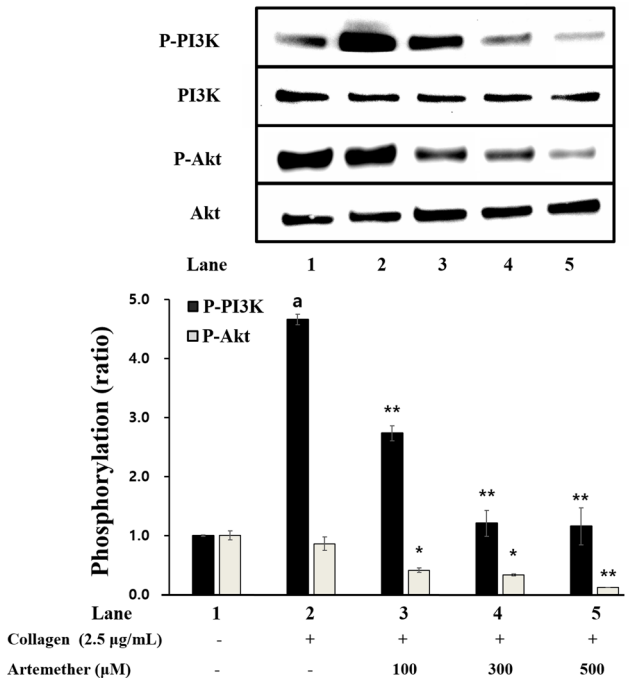

Fig. 3 Effects of artemether on PI3K and Akt phosphorylation. Western blotting was determined as described in "Materials and Methods". Data are expressed as mean ± SD (n =4). [a]p <0.05 compared with no-stimulated intact platelets, *p <0.05, **p <0.001 compared with the collagen-stimulated platelets

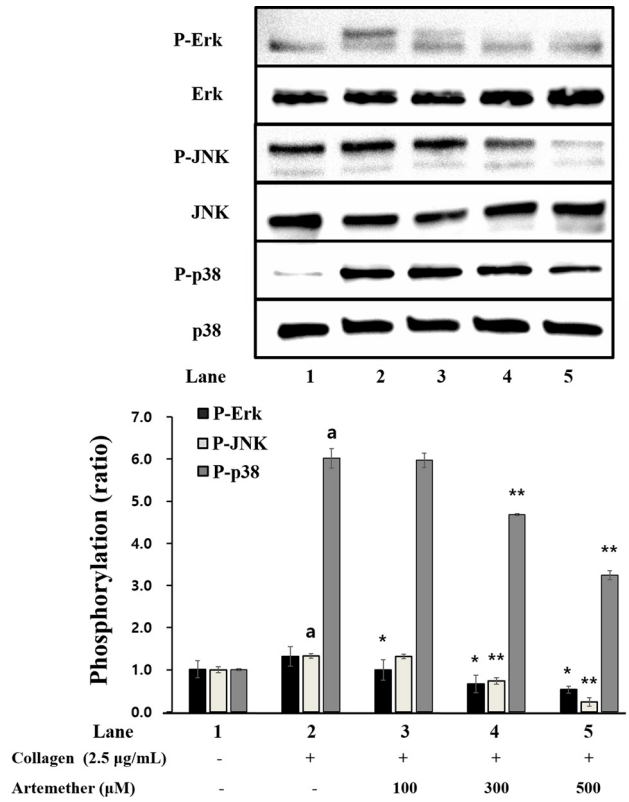

Fig. 4 Effects of artemether on MAPK phosphorylation. Western blotting was determined as described in "Materials and Methods". Data are expressed as mean ± SD (n =4). [a]p<0.05 compared with no-stimulated intact platelets, *p <0.05, **p <0.001 compared with the collagen-stimulated platelets

Statistical analyses

Analysis of variance (ANOVA) determined whether there were any differences considered significant amid the groups. Upon finding any differences that were significant, Scheffe's post hoc test was used using the SPSS V20.0 software from SPSS Inc in Chicago, Illinois (USA). Mean ± standard deviation represented the results and observations. Any P values less than 0.05 were considered significant (statistically).

Results and Discussion

Effect of artemether on collagen-induced human platelet aggregation

Any effect from artemether on human platelets induced by collagen and the inhibition of their aggregation was investigated. To induce platelet aggregation, collagen (2.5 μg/mL) was introduced, and aggregation confirmation of platelets was strong at 84.3±4.6% by collagen. However, it was confirmed that artemether strongly inhibited induced aggregation levels of platelets and inhibition was related to the concentration of artemether (50, 100, 300, 500 μM) (Fig. 2A). When the half-maximal inhibitory concentration (IC50) of artemether was checked, it was established at 157.92 μM (Fig. 2B), and it was confirmed by the released LDH whether artemether was cytotoxic to platelets. As a result, no significant changes were observed in human platelets due to the treatment of artemether (50, 100, 300, 500 μM) (Fig. 2C). According to these results, artemether is able to inhibit platelet aggregation in platelets induced with collagen and without cytotoxicity to platelets.

Effects of artemether on PI3K/Akt and MAPK pathways

In this study, we investigated the relationship of artemether and the phosphorylation of proteins PI3K/Akt. These are phospho-proteins shown to play a role in the secretion of granules within platelets. Demonstrated with Fig. 3, phosphorylation of PI3K/Akt proteins was in fact inhibited with the addition of artemether and shown in a concentration-dependent manner. This clearly demonstrates artemether can be used as an inhibitor to regulate PI3K/Akt phosphorylation in collagen-induced platelets. In addition, it was checked whether artemether had an effect on phosphorylation of MAPK proteins. Demonstrated in Fig. 4, collagen increased MAPK pathway proteins (ERK, JNK, p38) phosphorylation significantly when compared to uninjured cells, and decreased according to concentrations of artemether. Based on these results, artemether regulates platelet signaling by inhibiting phosphorylation of MAPK pathway proteins, ERK, JNK and p38. Among phosphorylated proteins, the pathway of PI3K/Akt is also

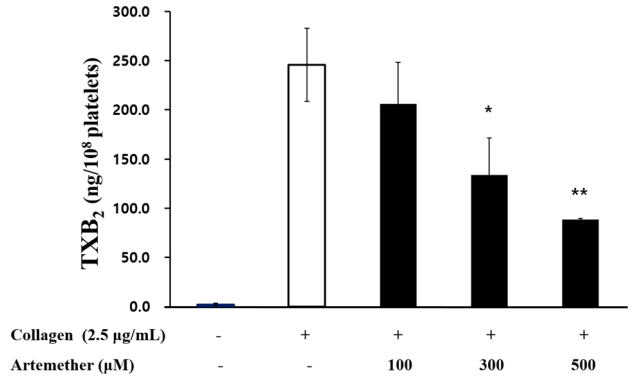

Fig. 5 Effects of artemether on TXB_2 production. Measurement of TXB_2 level was described in "Materials and Methods". Data are expressed as mean ± SD (n =4). ^{a}p <0.05 compared with no-stimulated intact platelets, *p <0.05, **p <0.001 compared with the collagen-stimulated platelets

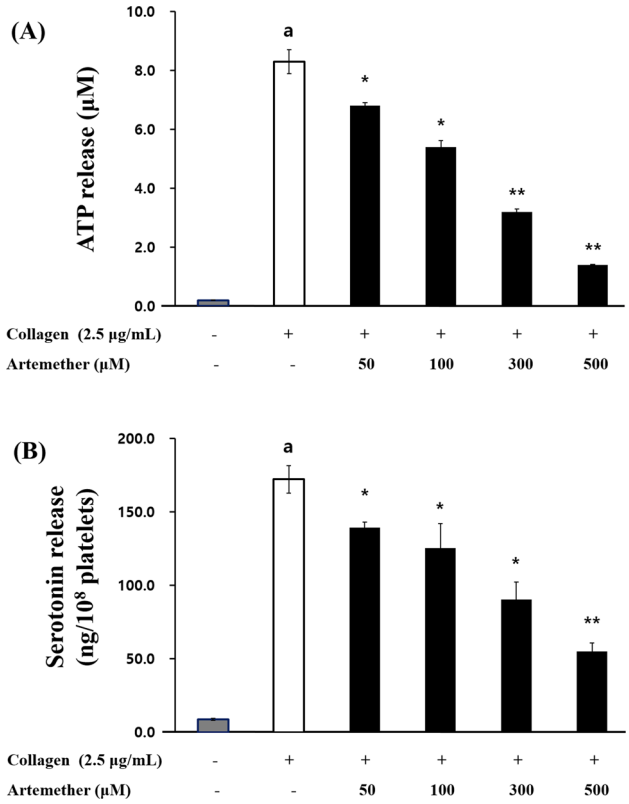

Fig. 6 Effects of artemether on granule secretion. (A) The effect of artemether on ATP release. (B) The effect of artemether on serotonin release. Measurement of ATP and serotonin release was described in "Materials and Methods". Data are expressed as mean ± SD (n =4). ^{a}p <0.05 compared with no-stimulated intact platelets, *p <0.05, **p <0.001 compared with the collagen-stimulated platelets

well known, and it has been demonstrated that their activation promotes dense granules secretion from platelets and plays a part in the regulation of platelet functions, including platelets aggregating [12]. In addition, phosphorylation of MAPK is known to contribute to platelets aggregating by causing platelet granule secretion [13,14]. In previous studies, it has been confirmed that MAPK is significantly present in human platelets, and it has been demonstrated that when platelets are activated by stimulants, they function by phosphorylation [15-17]. With these results, artemether inhibits platelet granulation and aggregation by inhibiting proteins in the PI3K/Akt pathway and MAPK pathway from being phosphorylated.

Effects of artemether on TXA_2 production

According to a previous study, it was shown phosphorylation of p38 was integral in platelets aggregating by affecting arachidonic acid secretion and TXA_2 production [18]. Since TXA_2 functions as a strong self-inducing agent that induces aggregation by additionally activating peripheral platelets, TXA_2 production is identified as an important indicator when evaluating substances exhibiting antiplatelet effects [18]. It is for the same reason that substances (aspirin/ozagrel) that inhibit TXA_2 production are being administered as agents with antiplatelet effects [19,20]. Furthermore, studies show MAPK phosphorylates phospholipase A_2 (PLA_2) in the cytoplasm and hydrolyzes phospholipids belonging to the membrane of platelets, and is involved in the production and secretion of arachidonic acid, thereby increasing TXA_2 production [21,22]. Therefore, it is necessary to check whether artemether, which inhibits MAPK and platelet aggregation, also affects any production of TXA_2.

As presentenced in Fig. 5, as a result of confirming the effect of artemether on the production of TXA_2, the level of TXA_2 production in cells (intact) came out to 2.37±1.14 ng/10^8 cells, which was increased up to 245.91±37.08 ng/10^8 cells when collagen was treated. However, the increased TXA_2 production was significantly decreased based on the concentration of artemether (100, 300, and 500 μM) (Fig. 5). This appears to be the result of inhibiting the secretion of arachidonic acid by inhibiting the phosphorylation of PLA_2 by MAPK, whose activity was reduced by artemether.

Effects of artemether on platelet granule secretion

Since granule release from platelets is important for amplifying platelet aggregation, we investigated whether artemether affects granule secretion of ATP and serotonin. As a result, it was confirmed that the ATP release amount of 0.23±0.01 μM from cells (intact) was strongly increased to 8.27±0.41 μM by collagen (2.5 μg/mL). However, the increase could be inhibited with varying concentrations of artemether (50, 100, 300 and 500 μM) (Fig. 6A). Also, it was confirmed that serotonin release was strongly increased from 7.38±1.99 ng/10^8 cells up to 173.90± 10.77 ng/10^8 cells by collagen (2.5 μg/mL). Yet, it was found that the collagen increased serotonin release was also reduced in a concentration-dependent manner to 139.27±3.78, 120.20±15.56, 90.35±11.79 and 54.79±5.87 ng/10^8 cells by artemether (50, 100, 300 and 500 μM). These results suggest that artemether strongly

inhibits platelets secreting granules and inhibits platelets from aggregating.

It is known that the secretion of granules within platelets is integral for a thrombus forming through inducing activation of platelets and promoting their adhesion and their aggregation in damaged blood vessels [23]. From these results, it was clarified that artemether was the reason for both platelet activation inhibition and platelet aggregation inhibition by concentration-dependently inhibiting ATP and serotonin released from dense granules in platelets by induction of collagen. Therefore, this study suggests that artemether contributed to antiplatelet action by inhibiting granule release through inhibition of the PI3K/Akt pathway and inhibiting TXA_2, a potent aggregation trigger, through inhibition of the MAPK signaling pathway.

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