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Research Article

Liensinine Inhibits Collagen-Induced Platelet Activation via PLCγ2–PKC–Ca^{2+} Signaling in Human Platelets

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Abstract

Background: *Nelumbo nucifera* (lotus) has been widely used in traditional Chinese medicine for centuries to treat various disorders, including cardiovascular diseases (CVDs), microbial infections, diabetes, and cancer. Liensinine, a natural alkaloid isolated from this plant, exhibits diverse pharmacological activities, including modulation of autophagy, induction of apoptosis in cancer cells, and cardioprotective effects in models of myocardial infarction. Since CVDs are closely associated with platelet activation, which plays a key role in arterial thrombosis, suppressing platelet activation may be a useful strategy for thrombotic diseases; however, the antiplatelet potential of liensinine remains to be fully elucidated. Therefore, this study was designed to evaluate the effects of liensinine on platelet activation in human platelets. **Materials and Methods**: Platelet aggregometry, a luciferin–luciferase assay, fluorescence analysis, flow cytometry, immunoblotting, confocal microscopy, scanning electron microscopy (SEM), and an *in vivo* thrombosis model were used in this study. **Results**: Liensinine inhibited collagen-induced platelet aggregation in a concentration-dependent manner and exerted a moderate inhibitory effect on thrombin-induced aggregation; however, no effects were observed for liensinine on arachidonic acid-induced aggregation. Moreover, liensinine did not increase lactate dehydrogenase release even at concentrations up to 160 μM, indicating an absence of platelet cytotoxicity. Liensinine also reduced collagen-induced Ca^{2+} mobilization, ATP release, and P-selectin expression. Mechanistically, liensinine attenuated collagen-stimulated phosphorylation of phospholipase C gamma 2 (PLCγ2) and protein kinase C (PKC). In contrast, liensinine did not inhibit platelet aggregation induced by phorbol 12,13-dibutyrate (PDBu), a direct PKC activator. Furthermore, SEM analysis showed that liensinine reduced collagen-induced filopodial elongation in platelets. In the animal experiment, liensinine significantly prolonged the occlusion time of thrombotic platelet plug formation in mesenteric vessels. **Conclusion**: These findings demonstrate that liensinine inhibits collagen-induced platelet activation by suppressing the PLCγ2–PKC signaling cascade, thereby reducing Ca^{2+} mobilization and granule secretion and attenuating platelet morphological changes and aggregation. Overall, the present results suggest that liensinine possesses antiplatelet and antithrombotic activity.

Keywords: liensinine; platelet activation; collagen; phospholipase C gamma 2–protein kinase C; thrombosis

1. Introduction

Cardiovascular diseases (CVDs) remain the leading cause of mortality worldwide, with arterial thrombosis representing a key pathological event underlying myocardial infarction and ischemic stroke [1]. After vascular injury, exposure to subendothelial matrix proteins provides an adhesive surface for circulating platelets, leading to platelet adhesion, activation, secretion, and aggregation, all of which contribute to thrombus formation [2]. Among platelet agonists, collagen is particularly important because collagen initiates early platelet activation via glycoprotein VI (GPVI)-dependent signaling pathways [3]. Upon GPVI engagement, downstream kinases such as spleen tyrosine kinase (Syk) are activated, leading to phosphorylation of phospholipase Cγ2 (PLCγ2) and subsequent generation of the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG) [4]. IP_3 induces calcium ion (Ca^{2+}) release from intracellular stores, whereas DAG activates protein kinase C (PKC). Together, these signaling events promote granule secretion and integrin $\alpha_{IIb}\beta_3$ activation, ultimately enhancing platelet aggregation [4,5]. Since the PLCγ2–PKC signaling cascade is central to collagen-

mediated platelet activation, targeting this pathway has been proposed as a potential strategy for preventing arterial thrombosis [3,4,5].

Natural compounds with multitarget pharmacological properties have attracted considerable interest as potential modulators of platelet function [6]. *Nelumbo nucifera* (lotus) has long been used in traditional Chinese medicine to manage various disorders, including CVDs, microbial infections, diabetes, and cancer. Liensinine, predominantly isolated from the seed embryo of this plant, has also been traditionally used to manage CVDs, cancer, and inflammatory conditions [7]. In cancer cells, liensinine can disrupt autophagic flux by impairing Ras-related protein Rab-7a (RAB7A) recruitment, thereby acting as an inhibitor of autophagy/mitophagy [8]. In addition, liensinine has been shown to regulate cellular metabolism by activating AMP-activated protein kinase (AMPK) and destabilizing hypoxia-inducible factor (HIF)-1α, thereby promoting a metabolic shift from glycolysis to oxidative phosphorylation and increasing reactive oxygen species production [9]. In gallbladder cancer, liensinine induces apoptosis and G_2/M-phase cell-cycle arrest through caspase activation and modulation of cell-cycle regulatory proteins, including cyclin B1 and cyclin-dependent kinase 1 [10]. Liensinine also exerts anti-inflammatory and antioxidant effects, associated with reduced NF-κB signaling and decreased expression of inflammation-related proteins, including cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [11]. Moreover, recent studies have demonstrated that liensinine confers cardioprotective effects against myocardial infarction by suppressing Wnt/β-catenin signaling, suggesting that liensinine has potential relevance to CVDs [12]. Collectively, these studies indicate that liensinine has broad pharmacological activity; however, whether liensinine modulates platelet activation remains unclear.

Since platelet activation is a key process in thrombus formation, this study aimed to investigate whether liensinine modulates platelet activation and examined the underlying signaling mechanisms. Thus, the present study aimed to (i) determine the effects of liensinine on platelet aggregation induced by various agonists, (ii) evaluate its impact on intracellular calcium ion (Ca^{2+}) mobilization and granule secretion, and (iii) examine its effects on key signaling pathways involved in platelet activation, including PLCγ2 and PKC signaling.

2. Materials and Methods

2.1 Reagents

Liensinine was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Type I collagen was obtained from Chrono-Log (Havertown, PA, USA). Arachidonic acid (AA), thrombin, dimethyl sulfoxide (DMSO), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Alexa Fluor 647-labeled goat anti-mouse IgG and Alexa Fluor 488-labeled goat anti-rabbit IgG were obtained from Abcam (Waltham, MA, USA). An anti-α-tubulin monoclonal antibody (mAb; catalog no. sc-32293) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-PLCγ2 polyclonal antibody (pAb; catalog no. AF3192) was purchased from Affinity (Cincinnati, OH, USA). A phospho-PKC substrate pAb (catalog no. 2261) was purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2 Washed Human Platelet Preparation

Healthy adult volunteers were recruited for blood donation after confirmation that each volunteer had avoided antiplatelet medications and nonsteroidal anti-inflammatory drugs for at least 2 weeks prior to sampling. Ethical approval was granted by the Institutional Review Board of Taipei Medical University (TMU-JIRB-N202512100). All participants signed written informed consent forms, and the study procedures followed the principles of the Declaration of Helsinki. Washed human platelets were prepared from platelet-rich plasma as previously described [4]. Briefly, prostaglandin E_1 (1 μM) was added during platelet washing to minimize *ex vivo* activation, and the final platelet pellet was resuspended in modified Tyrode's buffer (pH 7.4) containing 1 mM Ca^{2+}.

2.3 Platelet Aggregation Assay

Washed platelets were adjusted to 3.6×10^8 platelets/mL and pretreated with liensinine at the indicated concentrations (5–20 μM), or with vehicle control (0.1% DMSO). After incubation, platelet activation was induced using the specified agonists. Platelet aggregation was measured with a lumi-aggregometer. Light transmission was continuously recorded for 6 min, and the results were expressed as the percentage of aggregation relative to the vehicle control.

2.4 Lactate Dehydrogenase (LDH) Release Assay

Platelet cytotoxicity was evaluated by measuring LDH release using a commercially available LDH assay kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. After treatment with liensinine, the reaction mixture was centrifuged, and the supernatant was collected for LDH measurement. Absorbance was measured with a microplate reader, and the percentage of LDH release was calculated relative to the maximal-release control.

2.5 Intracellular Calcium ($[Ca^{2+}]_i$) Measurement

Intracellular Ca^{2+} mobilization was measured using Fura-2 AM as previously described [13]. Briefly, washed platelets were labeled with 5 μM Fura-2 AM (Molecular Probes, Eugene, OR, USA) and resuspended in Ca^{2+}-containing Tyrode's buffer after removal of excess dye. After pretreatment with liensinine (10 or 20 μM) or vehicle

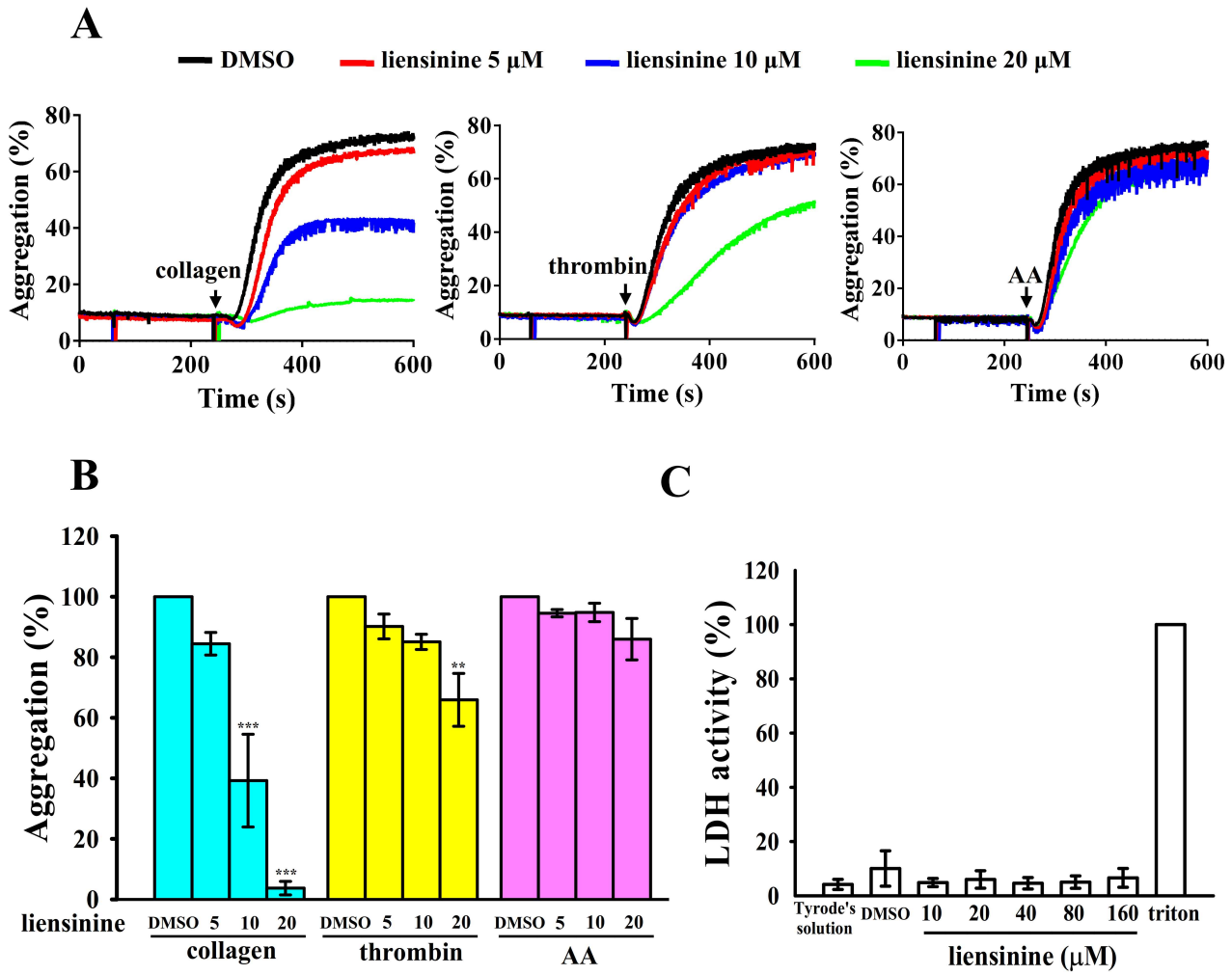

Fig. 1. Effects of liensinine on agonist-induced platelet aggregation and lactate dehydrogenase (LDH) release. (A) Washed human platelets were incubated with 0.1% dimethyl sulfoxide (DMSO) or liensinine (5–20 μM) for 3 min before stimulation with collagen (2 μg/mL), thrombin (0.02 U/mL), or arachidonic acid (AA) (60 μM) to assess platelet aggregation. (B) Concentration–response histograms of the inhibitory effects of liensinine on these agonist-induced responses (%). (C) LDH release was assessed after treatment with liensinine (10–160 μM) to evaluate cytotoxicity. Data are presented as the mean ± standard error of the mean (n = 4). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the 0.1% DMSO + agonist group.

control (0.1% DMSO), platelets were stimulated with collagen. Fluorescence was then measured using a spectrometer with alternating excitation at 340 nm and 380 nm and emission at 510 nm.

2.6 ATP Release Assay

Platelets were incubated with 20 μL of luciferase/luciferin reagent (Sigma, St. Louis, MO, USA) in the presence of liensinine (10 or 20 μM) or vehicle control (0.1% DMSO) for 3 min, and then stimulated with collagen. ATP release was monitored by recording chemiluminescence using a spectrometer. For quantitative analysis, ATP release was determined from the maximal chemiluminescence signal after collagen stimulation. The response in the collagen-stimulated vehicle control group (0.1% DMSO) was defined as 100%, and ATP release in the liensinine-treated groups was expressed as a percentage of this control response.

2.7 Analysis of P-selectin Expression

Following collagen stimulation, platelet suspensions were fixed and incubated in the dark with either FITC-conjugated anti-CD62P antibody (BioLegend, San Diego, CA, USA) or an isotype control. P-selectin expression was analyzed by flow cytometry. Platelets were gated based on their forward scatter (FSC) and side scatter (SSC) characteristics to exclude debris and non-platelet particles. FITC fluorescence was then analyzed within the gated platelet population. At least 10,000 platelet events were collected per sample. Results are expressed as mean fluorescence intensity (MFI).

2.8 Western Blot Analysis

Western blotting was conducted in accordance with our previously reported study [13]. Briefly, platelet proteins were separated, transferred to membranes, and immunodetected using the indicated primary antibodies at a dilution of 1:1000, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were visualized by enhanced chemiluminescence, and signal intensity was quantified by densitometry.

2.9 Evaluation of Platelet Morphology by Scanning Electron Microscopy (SEM)

Platelets were allowed to adhere to microscopy slides and were subsequently processed using standard electron microscopy preparation procedures, including aldehyde fixation, osmium tetroxide post-fixation, buffer washing, and graded ethanol dehydration. SEM was used to examine platelet surface morphology, and the spreading area was quantified from the acquired images using ImageJ software (version 1.52a; Wayne Rasband, National Institutes of Health, Bethesda, MD, USA; https://imagej.net/ij/).

2.10 Confocal Fluorescence Microscopy

Confocal immunofluorescence staining was performed as previously reported [13], with modifications to the primary antibodies used. Briefly, washed human platelets (3×10^7 cells/mL) were incubated with 0.1% DMSO or liensinine (20 μM), and then stimulated with collagen. The samples were processed for immunofluorescence staining using the indicated primary antibodies and fluorescence-conjugated secondary antibodies. Fluorescence signals were observed using a confocal laser scanning microscope equipped with a 100× oil immersion objective.

2.11 Thrombosis Model Induced by Sodium Fluorescein

All animal procedures were approved by the Institutional Animal Care and Use Committee of Taipei Medical University (LAC2026-0079). The study was carried out in accordance with the ARRIVE guidelines. A total of 24 six-week-old male C57BL/6 mice were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). Mice with body weights ranging from 20 to 25 g and were healthy. The mice were randomly assigned to three treatment groups and intraperitoneally administered 0.1% DMSO, liensinine (12 mg/kg), or aspirin (20 mg/kg), followed by tail vein injection of sodium fluorescein (15 μg/kg). After anesthesia with sodium pentobarbital (50 mg/kg, i.p.; 10 mg/mL solution prepared in sterile normal saline), mesenteric vessels (30–40 μm diameter) were exposed to irradiation to induce thrombus formation. The interval from the onset of irradiation to complete vessel occlusion was measured and defined as the occlusion time. No animals were excluded from the final analysis. After the experiment, mice were euthanized immediately by carbon dioxide inhalation at a displacement rate of 20–30% of the chamber volume/min. Death was confirmed by the absence of spontaneous respiration for more than 2 min and the loss of reflexes.

2.12 Statistical Analysis

Data are expressed as the mean ± standard error of the mean, and n represents independent experiments performed with platelets from different donors. Statistical analyses were carried out using SAS software (version 9.2; SAS Inc., Cary, NC, USA). Comparisons between multiple groups were performed using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls method. A p-value < 0.05 was considered statistically significant.

3. Results

3.1 Liensinine Suppresses Platelet Aggregation Induced by Various Agonists

The effects of liensinine on platelet aggregation were evaluated in collagen-, AA-, and thrombin-stimulated platelets. Liensinine (5–20 μM) most effectively inhibited collagen-induced aggregation (2 μg/mL) (Fig. 1A). At 20 μM, liensinine reduced collagen-triggered response by more than 80% compared with the 0.1% DMSO control group. In contrast, the inhibitory effect of liensinine on thrombin-induced aggregation was modest, reaching approximately 35% inhibition at 20 μM. Liensinine did not significantly affect AA-induced aggregation (Fig. 1B).

To determine whether the inhibitory effects of liensinine were associated with cytotoxicity, LDH release was measured. Liensinine did not significantly increase LDH release at concentrations up to 160 μM (Fig. 1C), indicating that the observed inhibitory effects were not attributable to cytotoxicity. Accordingly, collagen was used as the principal agonist in subsequent experiments, and liensinine at concentrations approximating the IC_{50} (10 μM) and the maximal inhibitory effect (20 μM) was used to investigate the underlying mechanisms associated with collagen-stimulated platelets.

3.2 Effects of Liensinine on Intracellular Ca^{2+} Mobilization, ATP Release, and P-Selectin Expression in Collagen-Activated Platelets

We further examined several key activation responses in collagen-stimulated platelets, including intracellular Ca^{2+} mobilization, ATP release, and P-selectin expression. Stimulation with collagen markedly increased intracellular Ca^{2+} mobilization, ATP release, and P-selectin surface expression in platelets (Fig. 2). Pretreatment with liensinine significantly attenuated these responses in a concentration-dependent manner. Liensinine at 10 and 20 μM reduced collagen-induced intracellular Ca^{2+} mobilization by approximately 45% and 51%, respectively (Fig. 2A). Consistently, liensinine significantly decreased collagen-induced ATP release (10 μM, 53.3 ± 14.0%; 20 μM, 20.2 ± 9.3%;

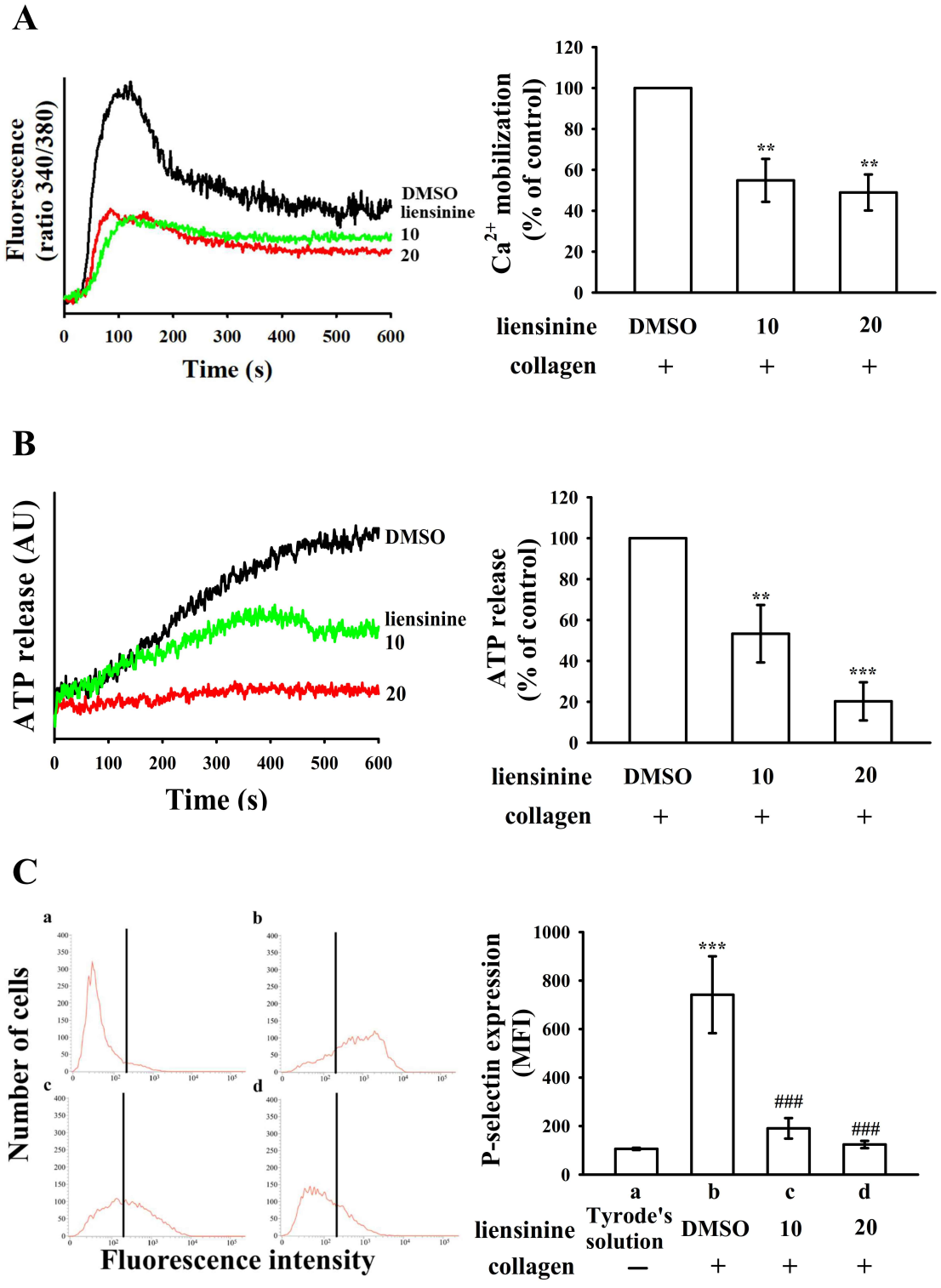

Fig. 2. Liensinine attenuates collagen-induced intracellular Ca^{2+} mobilization, ATP release, and P-selectin expression. Washed human platelets were incubated with 0.1% DMSO or liensinine (10 or 20 μM) before activation with collagen (2 μg/mL). The effects of liensinine on (A) intracellular Ca^{2+} mobilization, (B) ATP release, and (C) P-selectin surface expression (a) Tyrode's solution, (b) collagen-stimulated platelets, (c) liensinine 10 μM, and (d) 20 μM). Data are shown as the mean ± standard error of the mean (n = 4). (A,B) $^{**}p < 0.01$ and $^{***}p < 0.001$ versus 0.1% the DMSO + collagen group. (C) $^{***}p < 0.001$ versus the resting (Tyrode's solution) group; $^{###}p < 0.001$ versus the 0.1% DMSO + collagen group.

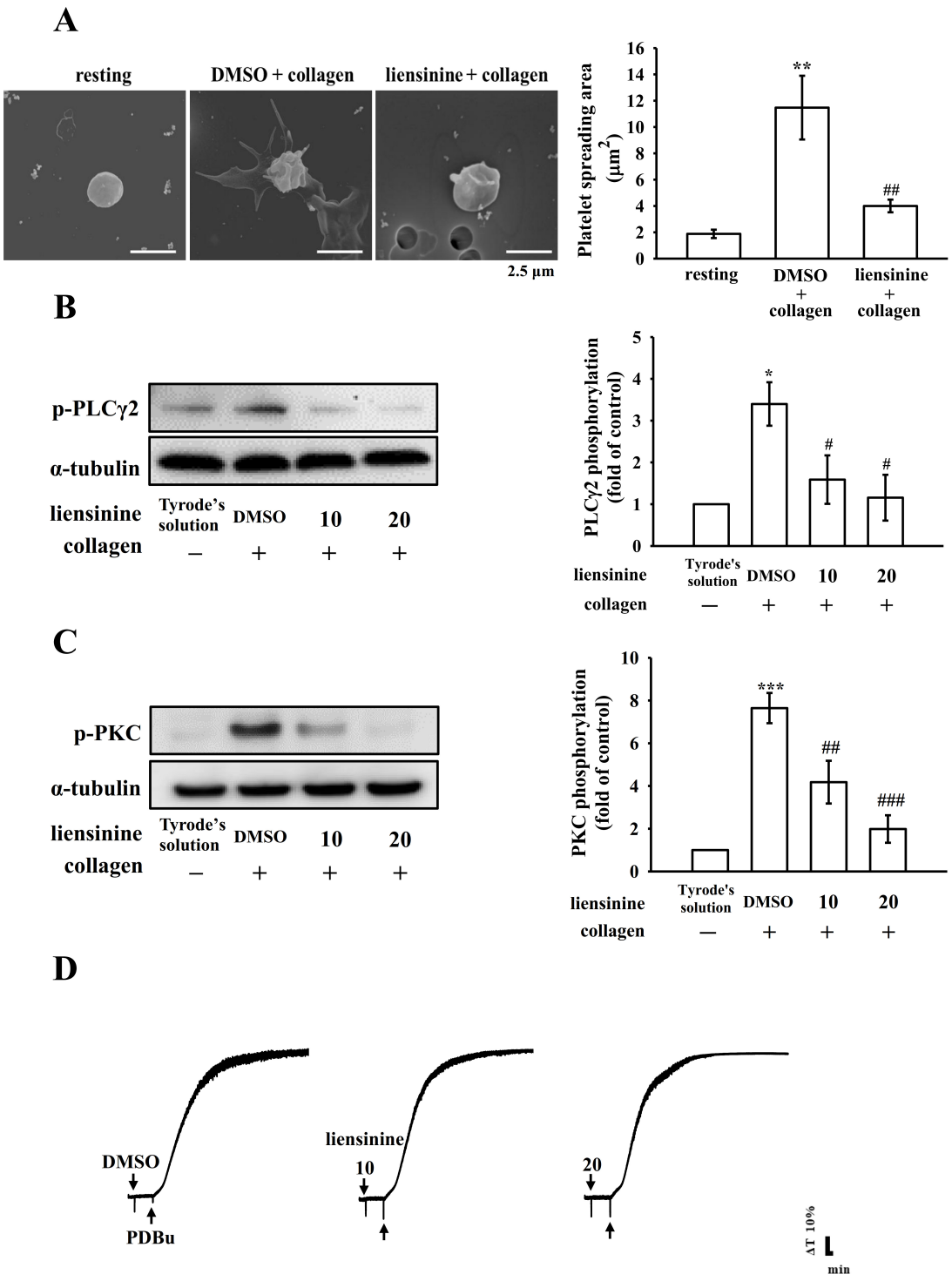

Fig. 3. Liensinine reduces collagen-induced morphological alterations and suppresses phospholipase C gamma 2 (PLCγ2)–protein kinase C (PKC) signaling in human platelets. (A) Platelet ultrastructure was examined by scanning electron microscopy (SEM) after treatment with 0.1% DMSO or liensinine (20 μM), followed by activation with collagen (2 μg/mL). Scale bar: 2.5 μm. (B,C) For Western blot analysis, phosphorylation of PLCγ2 and PKC was assessed in collagen-activated platelets treated with or without liensinine (10 or 20 μM). (D) Platelet aggregation induced by phorbol 12,13-dibutyrate (PDBu) was assessed after treatment with or without liensinine (10 and 20 μM). Data are shown as the mean ± standard error of the mean (n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the resting (Tyrode's solution) group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ versus the 0.1% DMSO + collagen group.

n = 4) compared with the 0.1% DMSO control group (Fig. 2B). In addition, flow cytometric analysis showed that P-selectin expression was significantly decreased from 741.7 ± 158.6 in the DMSO-treated group to 190.7 ± 42.3 and 124.0 ± 15.0 following treatment with 10 and 20 μM liensinine, respectively (Fig. 2C). These results demonstrate that liensinine effectively suppresses collagen-induced platelet activation by inhibiting these activation responses.

3.3 Attenuation of Platelet Morphological Changes by Liensinine

Platelet morphological changes were examined using SEM. Resting platelets exhibited a typical discoid shape with smooth surfaces and no obvious filopodia (Fig. 3A). Upon collagen stimulation, platelets underwent a marked morphological transformation, characterized by extensive shape change, formation of multiple pseudopodia, and platelet spreading. In contrast, pretreatment with liensinine (20 μM) markedly attenuated collagen-induced morphological changes, with platelets displaying fewer pseudopodia than those in the collagen-stimulated group. Quantitative analysis of the pronounced spreading area further showed that collagen significantly increased the platelet spreading area from 1.9 ± 0.3 μm^2 to 11.5 ± 2.4 μm^2, whereas liensinine reduced the collagen-induced spreading area to 4.0 ± 0.5 μm^2. These observations indicate that liensinine suppresses collagen-induced platelet activation and the associated morphological transformation.

3.4 Influence of Liensinine on PLCγ2–PKC Signaling and PDBu-Induced Platelet Aggregation

To investigate how liensinine affects collagen-induced platelet responses, we examined the PLCγ2–PKC signaling cascade. PLCγ2 is a key mediator of collagen-triggered platelet signaling that regulates downstream PKC activation [3]. Activation of PKC was determined by examining the phosphorylation level of p47 (pleckstrin), a major PKC substrate in platelets [14]. Collagen stimulation markedly increased PLCγ2 phosphorylation and enhanced downstream PKC activation (Fig. 3B,C). Pretreatment with liensinine significantly attenuated collagen-induced PLCγ2 phosphorylation and reduced phosphorylation of PKC substrates. To further determine whether liensinine directly interferes with PKC activation, platelets were stimulated with the direct PKC activator, phorbol 12,13-dibutyrate (PDBu). Liensinine had no significant effect on PDBu-induced platelet aggregation (Fig. 3D). As a positive inhibitory control for PKC-dependent responses, Gö 6983, a PKC inhibitor, was used in PDBu-stimulated platelet activation. Gö 6983 inhibited PDBu-induced platelet aggregation, whereas liensinine showed no significant inhibitory effect (**Supplementary Fig. 1A**). Gö 6983 also reduced PDBu-induced PKC phosphorylation, whereas liensinine did not suppress PDBu-induced PKC phosphorylation (**Supplementary Fig. 1B**). Confocal immunofluorescence analysis was then performed to visualize collagen-induced phosphorylation of PLCγ2 and PKC in human platelets (Fig. 4A,B). Collagen stimulation markedly increased the fluorescence intensity of phosphorylated PLCγ2 and PKC (green). Consistent with the immunoblotting data, pretreatment with liensinine (20 μM) substantially reduced collagen-induced phosphorylation signals of PLCγ2 and PKC. In contrast, the intensity of α-tubulin staining (red) did not differ notably among the groups (Fig. 4). These findings suggest that liensinine may suppress collagen-induced platelet activation, at least in part, by modulating the PLCγ2–PKC signaling pathway, rather than by directly inhibiting PKC activation.

3.5 Liensinine Attenuates Fluorescein-Induced Thrombosis in Mouse Mesenteric Vessels

The *in vivo* antithrombotic effect of liensinine was assessed using a fluorescein-induced thrombosis model in mouse mesenteric vessels. Following administration of liensinine (12 mg/kg), thrombotic occlusion was markedly delayed compared with the 0.1% DMSO control group (Fig. 5A). Aspirin served as a clinically established antiplatelet reference drug. Liensinine treatment significantly prolonged vessel occlusion time (0.1% DMSO: 169 ± 22 s; liensinine: 486 ± 57 s; n = 8; Fig. 5B), and a similar prolongation was observed in the aspirin-treated group (510 ± 49 s; n = 8; Fig. 5B). These results indicate that liensinine delays thrombotic platelet plug formation *in vivo* and exhibits antithrombotic activity comparable to aspirin under the present experimental conditions.

4. Discussion

The present study demonstrates that liensinine markedly inhibits platelet activation, a critical process involved in arterial thrombosis and CVDs [15,16]. Platelet-driven thrombus formation involves multiple activation events, including adhesion, secretion, aggregation, and cytoskeletal remodeling [16]. These processes have encouraged the search for natural compounds that can modulate platelet function and serve as candidates for antithrombotic drug development. Our results show that liensinine concentration-dependently reduced collagen-induced aggregation and produced a modest inhibitory response against thrombin-induced aggregation. However, liensinine did not significantly suppress AA-induced aggregation. Since AA is converted through COX-related enzymatic pathways to generate thromboxane A_2 (TXA_2), which activates thromboxane prostanoid receptors and amplifies platelet activation [17], these findings suggest that liensinine is unlikely to act through the AA–thromboxane pathway. To our knowledge, this is the first systematic study to evaluate the regulatory effects of liensinine on platelet activation and the associated signaling mechanisms in human platelets.

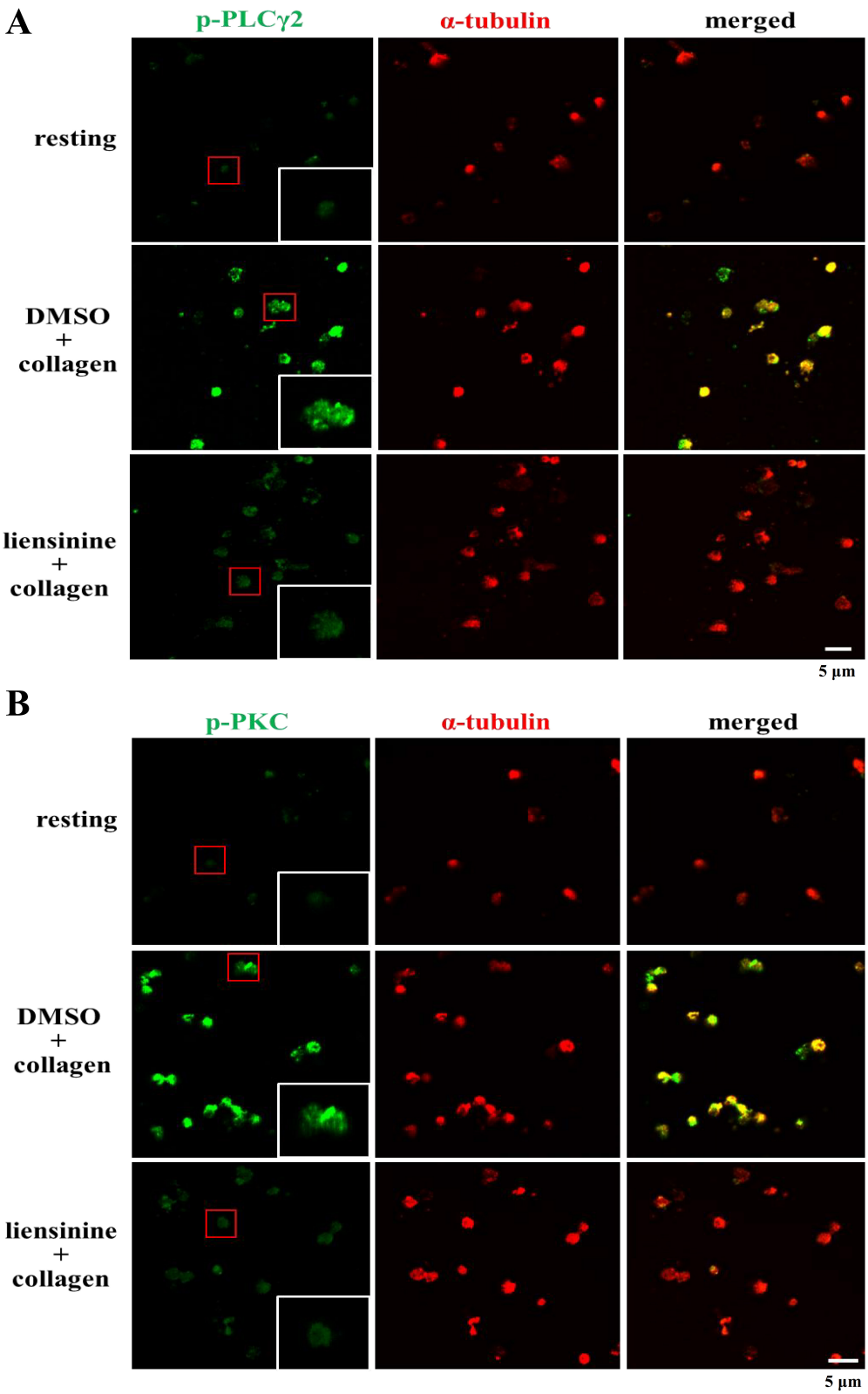

Fig. 4. Confocal immunofluorescence analysis of PLCγ2 and PKC phosphorylation after collagen stimulation. Washed human platelets were pretreated with 0.1% DMSO or liensinine (20 μM) and then stimulated with collagen (2 μg/mL). Platelets were fixed and subjected to immunofluorescence staining. Phosphorylated PLCγ2 (A) and PKC (B) were detected with specific antibodies and are shown in green, whereas α-tubulin is shown in red. Images are representative of four independent experiments. Scale bar: 5 μm.

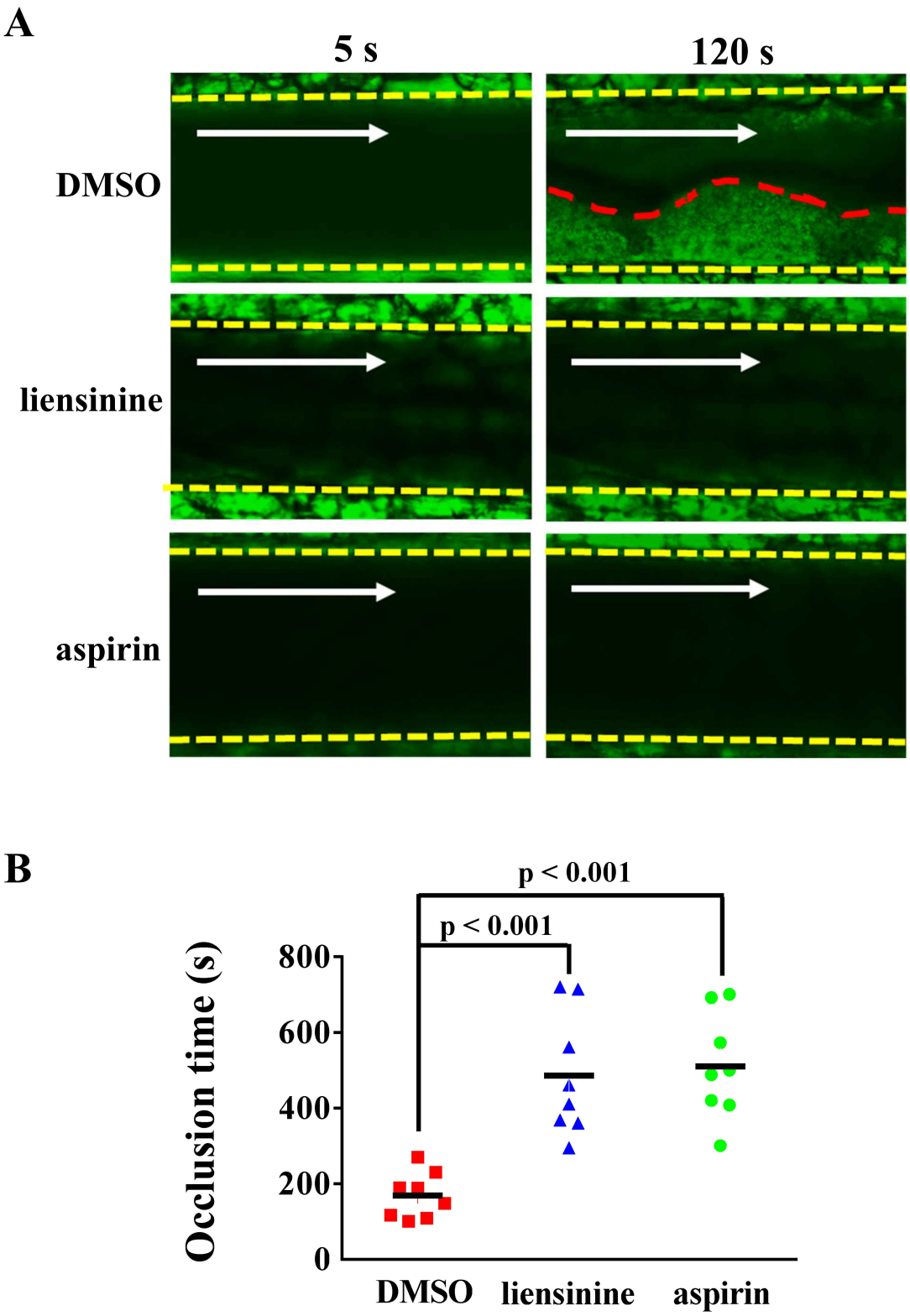

Fig. 5. Effect of liensinine on thrombus formation in mouse mesenteric vessels. (A) Mice received 0.1% DMSO, liensinine (12 mg/kg), or aspirin (20 mg/kg) by intraperitoneal injection. Mesenteric thrombosis was induced by fluorescein-mediated irradiation. Representative images were captured at 5 and 120 s after irradiation for each group. White arrows indicate the direction of blood flow, and the region below the red dashed line indicates the site of thrombus formation. (200× magnification). (B) Occlusion time was recorded. Each dot represents an individual mouse (n = 8). $p < 0.001$ versus the 0.1% DMSO group.

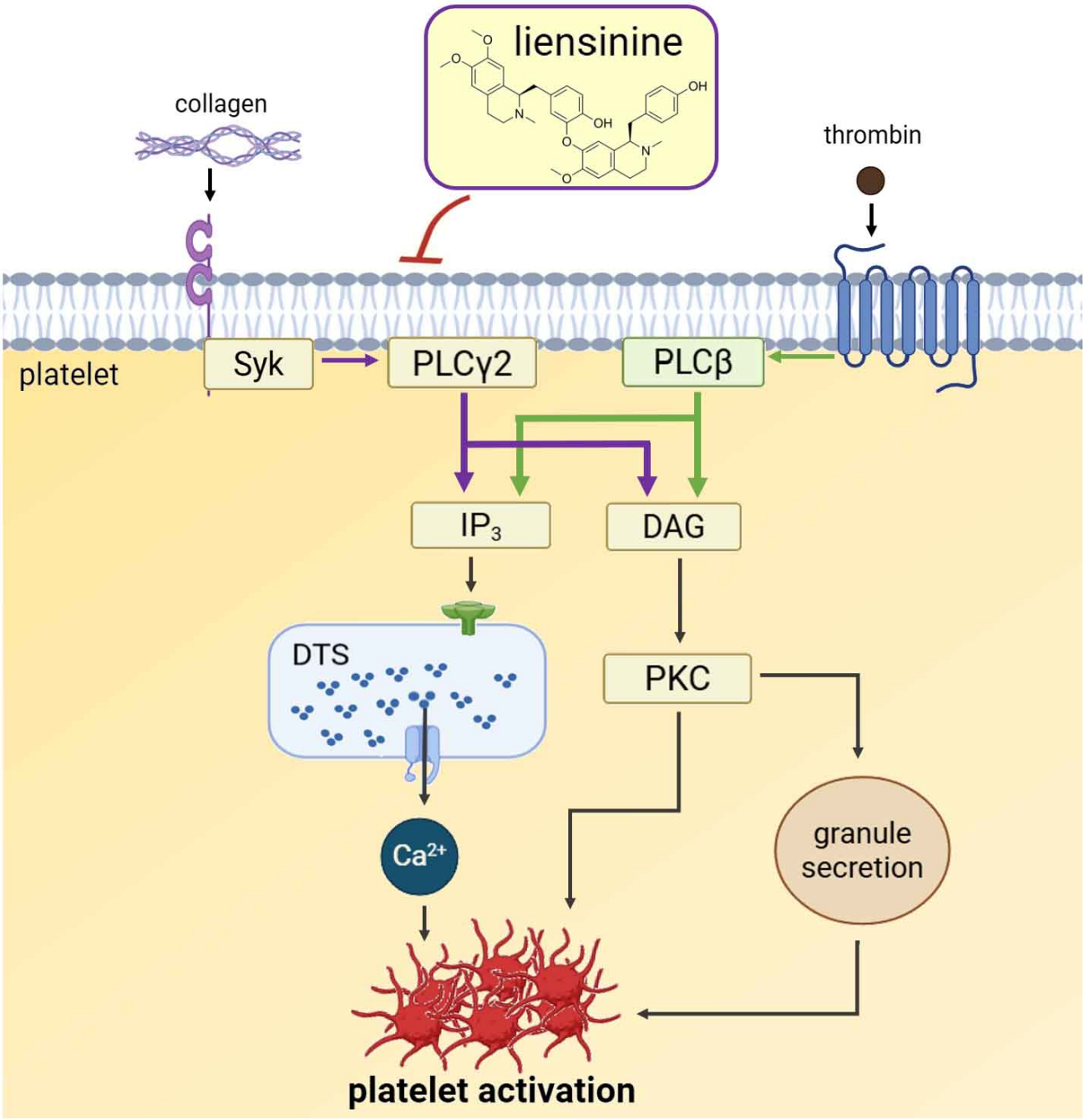

Fig. 6. Schematic illustration of the signaling pathway targeted by liensinine in collagen-activated platelets. Upon collagen stimulation, spleen tyrosine kinase (Syk) activation leads to PLCγ2-mediated generation of inositol trisphosphate (IP_3) and diacylglycerol (DAG), resulting in Ca^{2+} release from the dense tubular system (DTS) and PKC activation. Together, these events promote granule secretion and platelet activation. Liensinine suppresses the PLCγ2–PKC–Ca^{2+} signaling cascade in collagen-activated platelets, thereby attenuating platelet aggregation. Red inhibitory arrows indicate suppression, whereas black arrows represent canonical signaling pathways. Purple arrows denote collagen receptor-mediated signaling, and green arrows indicate thrombin receptor-related pathways.

Collagen is one of the most potent physiological agonists of platelet activation and initiates platelet signaling primarily through the GPVI receptor [3]. Meanwhile, activation of GPVI leads to phosphorylation of Syk and subsequent activation of PLCγ2, which represents a key signaling step in collagen-induced platelet activation [4]. Consistent with this mechanistic interplay, our data show that liensinine markedly suppressed collagen-induced PLCγ2 phosphorylation and subsequent PKC activation. PLCγ2-dependent signaling plays a crucial role in platelet activation by triggering intracellular Ca^{2+} mobilization and downstream secretion responses [18]. In agree-

ment with this concept, we observed that liensinine significantly reduced intracellular Ca^{2+} mobilization in collagen-stimulated platelets. Elevation of intracellular Ca^{2+} is a central event in platelet activation and is essential for granule secretion, integrin activation, and cytoskeletal rearrangement [18]. Collectively, these results suggest that liensinine attenuates collagen-triggered platelet activation by downregulating the PLCγ2–PKC signaling pathway, accompanied by reduced Ca^{2+} mobilization and granule release, thereby inhibiting platelet aggregation (Fig. 6).

Granule secretion represents an important amplification mechanism during platelet activation. Upon stimulation, platelets initiate intracellular signaling events that elevate cytosolic Ca^{2+} levels and promote exocytosis of storage granules containing bioactive molecules. Dense granules predominantly release small soluble molecules, such as ADP and ATP [19], which amplify platelet activation by recruiting additional platelets and strengthening aggregation. In contrast, α-granules serve as storage sites for multiple membrane-bound and soluble proteins, including P-selectin and fibrinogen, which contribute to platelet adhesion and thrombus stabilization [20]. Surface exposure of P-selectin after α-granule secretion is commonly used as a marker of platelet activation [20]. In the present study, liensinine markedly attenuated collagen-induced ATP release (Fig. 2B) and P-selectin expression (Fig. 2C), indicating that this compound suppresses both dense-granule and α-granule secretion during platelet activation.

Activation of PLC represents a critical step in platelet signal transduction. Meanwhile, human platelets express two major PLC isoforms, PLCβ and PLCγ, which participate in distinct signaling pathways depending on the agonist involved [21]. The PLCγ family comprises two isoforms, PLCγ1 and PLCγ2; notably, PLCγ2 plays a central role in collagen-induced platelet activation via GPVI-dependent signaling in human platelets [21]. In contrast, thrombin activates platelets primarily through protease-activated receptors (PAR1 and PAR4) coupled to Gαq proteins, thereby activating PLCβ [21,22]. These mechanistic differences may partly explain why certain compounds strongly inhibit collagen-induced platelet aggregation while exerting only modest effects on thrombin-mediated platelet activation. Consistent with this interpretation, our results show that liensinine had no inhibitory effect on PDBu-triggered platelet aggregation. Since PDBu directly activates PKC and bypasses receptor-mediated signaling, this observation indicates that liensinine does not function as a direct PKC inhibitor. Gö 6983 suppressed PDBu-induced platelet aggregation and PKC phosphorylation, whereas liensinine did not significantly affect either response (**Supplementary Fig. 1**). Thus, the inhibitory action of liensinine likely occurs upstream of PKC activation, as evidenced by the observed suppression of PLCγ2 phosphorylation in collagen-stimulated platelets. In addition to PLCγ2–PKC signaling, PI3K and p38 MAPK are important signaling molecules involved in platelet activation [23,24]. Our results further showed that liensinine attenuated collagen-induced phosphorylation of p38 MAPK, but not PI3K (**Supplementary Fig. 2**), suggesting that inhibition of p38 MAPK signaling may also contribute to the suppressive effect of liensinine on collagen-induced platelet activation. This result is consistent with previous evidence suggesting that PLCγ2–PKC signaling may contribute to the regulation of p38 MAPK activation in platelets [25]. Nevertheless, the possibility that liensinine influences additional signaling pathways that contribute to platelet activation remains, although further clarification is required.

Platelet morphological transformation is a hallmark of platelet activation and reflects dynamic cytoskeletal remodeling during the transition from resting discoid platelets to fully activated cells. Upon stimulation, platelets rapidly reorganize their actin and microtubule cytoskeletons, leading to a spherical shape accompanied by filopodia extension, which increases surface area and facilitates adhesion and aggregation during thrombus formation [26,27]. These structural changes are closely associated with intracellular signaling events and granule secretion, both of which contribute to the stabilization and growth of platelet aggregates [16]. Consistent with these concepts, our SEM observations revealed that collagen stimulation induced pronounced morphological alterations characterized by filopodia extension and extensive platelet spreading, indicative of robust platelet activation. In contrast, platelets treated with liensinine retained a relatively rounded morphology and exhibited markedly reduced filopodia elongation. These findings suggest that liensinine interferes with cytoskeletal reorganization, further supporting the inhibitory effect of liensinine on platelet activation.

Animal models of vascular thrombosis are valuable for examining the *in vivo* antithrombotic activity of test compounds. In the fluorescein sodium-induced thrombosis model, continuous irradiation of mesenteric vessels causes endothelial injury and exposes the underlying collagen matrix, thereby promoting platelet adhesion and aggregation at the injury site and leading to thrombus formation [17]. In our study, administration of liensinine at 12 mg/kg significantly delayed vessel occlusion, suggesting that liensinine exerts antithrombotic effects *in vivo*. Thus, liensinine may be a promising candidate for further investigation in thromboembolic disorders.

5. Limitations

The precise upstream molecular target of liensinine in collagen-induced platelet activation was not fully determined. Although liensinine reduced collagen-induced PLCγ2 phosphorylation and downstream platelet activation responses, the present data do not establish whether liensinine directly targets PLCγ2 or modulates upstream GPVI-related signaling. Moreover, since Syk phosphorylation was not examined, further studies assessing Syk

and other GPVI-proximal signaling molecules are needed to clarify the precise molecular target of liensinine. In addition, bleeding risk was not evaluated in the present study. Liensinine significantly prolonged occlusion time in the fluorescein-induced mesenteric vessel thrombosis model; this result supports the antithrombotic activity of liensinine *in vivo* but does not establish any associated effects on hemostasis. Therefore, future studies should evaluate bleeding time to improve the assessment of the therapeutic potential and safety profile of liensinine.

6. Conclusion

Our findings indicate that liensinine attenuates collagen-triggered platelet activation by suppressing the PLCγ2–PKC signaling pathway. This effect is associated with decreased Ca^{2+} mobilization and reduced granule secretion, as reflected by lower ATP release and P-selectin expression, ultimately leading to inhibition of platelet aggregation (Fig. 6). In addition, SEM analysis revealed that liensinine markedly reduced collagen-induced morphological changes in platelets. Collectively, these findings suggest that liensinine possesses antiplatelet and antithrombotic activity. However, the possibility that liensinine may also affect additional signaling pathways involved in platelet activation cannot be excluded and should be addressed in future studies.

Availability of Data and Materials

All data generated or analyzed during this study are included in this article. The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Conceptualization, CCChang, CYH and CWH; methodology, CCChang and KLL; investigation, CCChang, KLL, YC, CCChiu, AGD and JRS; formal analysis, CCChang and KLL; writing—original draft preparation, CCChang, KLL, CYH and CWH; writing—review and editing, YC, CCChiu, AGD and JRS; supervision, CWH; funding acquisition, CYH. All authors have read and agreed to the published version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was carried out in accordance with the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Taipei Medical University (TMU-JIRB-N202512100). Written informed consent was obtained from all participants before blood collection. All animal procedures were approved by the Institutional Animal Care and Use Committee of Taipei Medical University (LAC2026-0079) and were performed in accordance with the approved institutional guidelines for the care and use of laboratory animals.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/IJP52068.

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