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Metformin Serves as a Novel Drug Treatment for Arterial Thrombosis: Inhibitory Mechanisms on Collagen-Induced Human Platelet Activation

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Abstract: Metformin is widely used as first-line medication for type 2 diabetes (T2D), the main disease comorbid with kidney disease, cardiovascular diseases (CVDs), and retinopathy. Platelets are crucial in platelet-dependent arterial thrombosis, which causes CVDs and cerebrovascular diseases. Research indicates that metformin may improve these diseases; metformin reportedly reduced platelet activation in rats. However, no reports have included human platelets. We investigated the mechanisms underlying metformin's effects on platelet activation by using human platelets and evaluated its in vivo effectiveness in experimental mice. Metformin inhibited platelet aggregation stimulated by collagen but not by arachidonic acid, U46619, or thrombin. Metformin suppressed ATP release, $[Ca^{2+}]i$ mobilization, and P-selectin expression, as well as phospholipase C (PLC)γ2/protein kinase C (PKC), p38 mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K)/Akt/glycogen synthase kinase-3β (GSK3β) phosphorylation. Metformin did not affect vasodilator-stimulated phosphoprotein (VASP) phosphorylation. In the animal studies, metformin reduced acute pulmonary thromboembolism mortality without increasing bleeding times. These results provide insights into the role and mechanisms of metformin in human platelet activation. Metformin decreased platelet activation by interfering with the PLCγ2/PKC, PI3K/Akt/GSK3β, and p38 MAPK pathways through a VASP-independent mechanism. Metformin demonstrates promise as a new class of antiplatelet agent that can inhibit platelet activation.

Keywords: human platelets; metformin; p38 MAPK; PLCγ2; pulmonary thrombosis; PI3K/Akt/GSK3β; type 2 diabetes

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1. Introduction

Type 2 diabetes (T2D), the most common type of diabetes, affects more than 462 million people globally, or 6.28% of the world's population [1]. T2D is the main endocrine driver for the global burden of disease and has become the ninth leading cause of death [2]. Patients with T2D typically have several comorbid diseases, such as chronic kidney disease, hypertension, cardiovascular diseases (CVDs), retinopathy, neurodegenerative diseases, and cancers [3]. Among these, CVDs and chronic kidney disease are the leading causes of

mortality in those with T2D [3]. Metformin has long been used as first-line medication for T2D because it is safe and inexpensive; it is the most widely prescribed drug for treating T2D [4]. Metformin is a biguanide derivative that reduces blood glucose levels through its ability to regulate energy metabolism; this regulation involves inhibiting hepatic gluconeogenesis, reducing glucose absorption, and elevating glucose utilization in peripheral tissues [5]. Numerous studies have revealed that metformin can improve inflammation, aging-related diseases, obesity, CVDs, chronic kidney disease, inflammatory bowel disease, cancers, polycystic ovarian syndrome, osteoporosis, and periodontitis [5].

Platelets, anucleated blood cells, participate in normal hemostasis process and arterial thrombosis, which induces various CVDs and cerebrovascular diseases. The event of platelet adherence and aggregation are thought to be involved in initiating intraluminal thrombosis. While endothelial damage occurs in blood vessels, platelets adhere to the disrupted surface, and subsequently release active biological substances, thus triggering aggregation of the platelets [6]. Platelets are activated by a variety of physiological agonists (e.g., collagen, thrombin, ADP, epinephrine, and PAF). These agonists are thought to exert their effects by interacting with specific receptors on the platelet membranes. The primary effects of agonists may be enhanced by secondary effects caused by the synthesis of thromboxane A_2 (TxA_2) from the arachidonic acid (AA) or by the secretion of ADP from the dense granules in platelets. ADP binds to two major purinergic receptors ($P2Y_1$ and $P2Y_{12}$), which play an important role in potentiating platelet activation induced by other aggregating agonists [7]. Drugs that can inhibit platelet activation can also greatly diminish the danger of critical events, such as vascular death, myocardial infarction, and ischemic stroke, especially in patients with atherosclerotic vascular diseases. Clinical trials have demonstrated the beneficial effects of metformin on CVDs; in one study, metformin reduced mortality and diabetes-associated thrombotic complications, such as endothelial dysfunction (ED), myocardial infarction, acute myocarditis, and chronic heart failure [3,8]. Metformin can directly protect injury-induced ED, which is beyond its glucose-lowering effects [9]. Metformin markedly diminished rat platelet activation through decreasing the extracellular mitochondrial DNA release, protection of mitochondrial function, and thereby reducing activated platelet-induced mitochondrial hyperpolarization-associated membrane damage [10]. Furthermore, metformin treatment could inhibit platelet aggregation in platelet-rich plasma (PRP) from the healthy human subjects [11] or type I diabetes patients [12]. Additionally, metformin was reported to decrease in vivo oxidative stress and reduce platelet activation through increasing plasma vitamin A and E or decreasing platelet superoxide anion levels in T2D patients [13,14]. Together, metformin may, thus, have potential as a therapeutic agent for arterial thrombosis. However, relatively few studies investigated the detailed mechanisms of metformin in platelet activation. Therefore, this study investigated the underlying mechanisms of metformin in human platelets and further evaluated the in vivo effectiveness of metformin in experimental mice.

2. Materials and Methods

2.1. Chemicals and Reagents

Metformin, collagen (type I), luciferin–luciferase, AA, 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin (U46619), heparin, prostaglandin E_1 (PGE_1), nitroglycerin (NTG), phenylmethylsulfonyl fluoride, sodium orthovanadate, sodium pyrophosphate, aprotinin, leupeptin, sodium fluoride, phorbol 12,13-dibutyrate (PDBu), bovine serum albumin (BSA), and thrombin were purchased from Sigma Aldrich (St. Louis, MO, USA), and anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}) polyclonal antibody (pAb) was purchased from Affinity (Cincinnati, OH, USA). Anti-phospho-Jun N-terminal kinase (JNK) (Thr^{183}/Tyr^{185}), anti-SAPK/JNK, anti-phospho-(Ser) protein kinase C (PKC) substrate, and anti-phosphophosphoinositide 3-kinase (PI3K) p85 (Tyr^{458})/p55 (Tyr^{199}) pAbs and anti-Akt, anti-p38 mitogen-activated protein kinase (MAPK), anti-phospholipaseγ2 (PLCγ2), and anti-PI3K p85 (19H8) monoclonal antibodies (mAbs) were purchased from Cell Signaling (Beverly, MA, USA). Anti-phospho PLCγ2 mAb was obtained from Abcam (Cambridge, UK). Anti-

phospho glycogen synthase kinase-3α/β (GSK3α/β) and anti-GSK3α/β mAbs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-Akt (Ser^{473}) pAb was purchased from BioVision (Mountain View, CA, USA). Anti-vasodilator-stimulated phosphoprotein (VASP) (phospho Ser^{157}), anti-VASP (phospho Ser^{239}), anti-VASP, anti-pleckstrin, and extracellular signal–regulated kinase (ERK) 1 (phosphate Thr^{202}/Tyr^{204}) + ERK2 (phosphate Thr^{185}/Tyr^{187}) pAbs were purchased from GeneTex (Irvine, CA, USA). CF™488A Dye and CF™405M Dye were obtained from Biotium (Hayward, CA, USA). Bio-Rad Laboratories (Hercules, CA, USA) and Molecular Probes (Eugene, OR, USA) supplied protein assay dye reagent concentrate and Fura 2-AM, respectively. The FITC-anti-human CD42P (P-selectin) mAb was obtained from BioLegend (San Diego, CA, USA). H89 and KT5823 were purchased from Cayman Chem (Ann Arbor, MI, USA). The stock solution of metformin (100 mM) was dissolved in phosphate-buffered saline (PBS) and stored at 4 °C until use.

2.2. Platelet Aggregation, ATP Release Reaction, and Lactate Dehydrogenase Assay

After the confirmation of all procedures in this study by directives of the Declaration of Helsinki, the Institutional Review Board of Taipei Medical University (TMU-JIRB-N201812024) was further approved. All human volunteers who participated in this study signed a consent form. Human platelet suspensions were prepared as previously described [15]. Blood was collected from 35 healthy human volunteers (age: 20–35 years) who had taken no medication during the 2 weeks preceding collection. The blood was mixed with an acid-citrate-dextrose (9:1, *v*/*v*) solution. After centrifuging for 10 min at 120× *g*, the resultant supernatant (PRP) from the centrifugal samples, was supplemented with EDTA (2 mM) and heparin (6.4 U/mL) for 5 min and, again, subject to centrifugation at 500× *g* for 10 min. A 5 mL Tyrode's solution was used to resuspend the obtained platelet pellet and kept for 10 min at 37 °C. After the spin at 500× *g* for 10 min, the washing procedure was repeated. The washed platelets were suspended in Tyrode's solution that contained BSA (3.5 mg/mL). A Coulter counter (Beckman Coulter, Miami, FL, USA) was applied to count platelet numbers. The final Ca^{2+} concentration was 1 mM in the Tyrode's solution.

According to the method described by Chen et al. [15], the platelet aggregation was measured using Lumi-Aggregometer (Payton Associates, Scarborough, ON, Canada). Before adding various agonists, namely collagen, AA, U46619, and thrombin, to the platelet suspensions (3.6×10^8 cells/mL), they were preincubated with various concentrations of metformin (1.2–1.8 mM) or an isovolumetric solvent control (PBS) for 3 min. The reaction was allowed to proceed for 6 min, and the extent of aggregation was expressed in light transmission units. To measure the release of ATP, 20 μL of a luciferin–luciferase mixture was added 1 min before agonists were added, and the amount of ATP released in the experimental group was compared with that of the control by using an F-7000 spectrometer (Hitachi, Tokyo, Japan) according to the manufacturer's instructions. Moreover, washed platelets were preincubated with different concentrations of metformin (2–10 mM) or PBS for 20 min at 37 °C. An aliquot of the supernatant (10 μL) was deposited on a Fuji Dri-Chem slide (LDH-PIII; Fuji, Tokyo, Japan), and absorbance was read at a wavelength of 540 nm. The highest lactate dehydrogenase (LDH) release was observed in the positive control triton (0.5%)-treated platelets.

2.3. Intracellular $[Ca^{2+}]i$ Mobilization and FITC-P-Selectin Expression in Human Platelets

The supernatant that was collected from the centrifuged citrated whole blood was incubated with Fura 2-AM (5 μM) for 1 h. As mentioned in Section 2.2, human platelets were prepared and they were adjusted to have 1 mM Ca^{2+}. The relative intracellular Ca^{2+} ion concentration ($[Ca^{2+}]i$) was measured with excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm [16]. In addition, washed platelets at a density of 3.6×10^8 cells/mL were preincubated with PBS or metformin (1.2 and 1.8 mM) and FITC-conjugated anti-P-selectin mAb (2 μg/mL) for 3 min and were then stimulated with collagen (1 μg/mL). Subsequently, fluorescein-labeled platelets were identified in the

suspensions by using a flow cytometer (FAC Scan system; Becton Dickinson, San Jose, CA, USA). Data were generated almost from 50,000 platelets in each experimental group, and the platelets were recognized according to their forward and orthogonal light-scattering characteristic profiles.

2.4. Immunoblotting

The platelet suspensions (1.2×10^9 cells/mL) were initially treated with metformin (1.2 and 1.8 mM), PBS, PGE_1 (20 nM), or NTG (10 μM), and collagen (1 μg/mL) was subsequently added to provoke platelet activation. After the reaction was terminated, platelets were directly resuspended in 200 μL of a lysis buffer (HEPES 50 mM, pH 7.4, NaCl 50 mM, 1% Triton X-100, and EDTA 5 mM supplemented with leupeptin 2 μg/mL, aprotinin 10 μg/mL, sodium pyrophosphate 5 mM, NaF 10 mM, sodium pyrophosphate 5 mM, PMSF 1 mM, and sodium orthovanadate 1 mM) for 2 h. Lysates with 60 μg of protein were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved proteins were transferred onto PVDF membranes and they were blocked using TBST (10 mM Tris-base, 0.01% Tween 20, and 100 mM NaCl) containing 5% BSA for 1 h. Protein concentrations were calculated using the Bradford protein assay (Bio-Rad). The proteins of interest were identified by using their respective primary antibodies (diluted 1:1000 in TBST) for 2 h. The optical density of the protein bands was quantified using a video densitometer and Bio-profil Biolight software, version V2000.01 (Vilber Lourmat, Marne-la-Vallée, France). The relative expression of targeted proteins was calculated after being normalized to their respective total proteins.

2.5. Immunofluorescence Staining Assay

A 4% (*v*/*v*) paraformaldehyde was used to fix the resting or collagen-activated platelets in poly-L-lysine-coated coverslips for 1 h, and they were exposed to 0.1% Triton X-100 and then incubated with 5% BSA for another 1 h. The coverslips were then stained with either the anti-phospho PLCγ2 mAb, phospho PKC substrate pAb, or α-tubulin mAb (all diluted 1:100 in 5% BSA) for 24 h and with goat anti-rabbit CF™ 488A Dye or anti-mouse CF™ 405M Dye (all diluted 1:200 in 5% BSA) for 1 h to detect PLCγ2 and PKC phosphorylation under confocal microscopy (Leica TCS SP5, Mannheim, Germany) with a 100× oil immersion objective lens.

2.6. Measurement of Pulmonary Microvascular Thrombosis in Mice

Acute pulmonary microvascular thrombosis was induced by using a formerly defined method [17]. All procedures in this study were conducted with an affidavit of approval of our animal use protocol from Taipei Medical University (LAC-2020-0294). The total of 60 mice were arbitrarily separated into five groups, with 12 mice in each group. Male ICR mice (6 weeks) were intraperitoneally injected with metformin (150 and 250 mg/kg) or PBS. After 5 min, ADP (0.7 mg/g) was injected into the mice's tail veins. The mortality rate of mice was evaluated 10 min after injection.

2.7. Mouse Tail Bleeding Assay

To this tail bleeding assay, 32 male ICR mice were arbitrarily separated into four groups, with eight mice in each group. The mice were anaesthetized and metformin (150 and 250 mg/kg) or PBS were intraperitoneally administered for 30 min. Following total amputation of tip of the tails, they were openly positioned into normal saline at 37 °C. The bleeding time was monitored until the bleeding clogged totally.

2.8. Statistical Analysis

The results are expressed as mean ± standard error of the mean, and *n* refers to the number of experiments conducted in this study. Differences among the experimental groups were analyzed using one-way analysis of variance (ANOVA), with the Student–Newman–Keuls method employed as a post hoc test. In addition, differences between

groups were assessed for statistical significance using the Fisher's exact test for the study of pulmonary microvascular thrombosis in mice. $p < 0.05$ indicated significance. SAS Version 9.2 (SAS, Cary, NC, USA) was applied for analyzing statistical evaluation.

3. Results

3.1. Mitigation of Human Platelet Aggregation by Metformin

As previously reported [10], metformin (1 mM) significantly diminished platelet aggregation stimulated by ADP (20 μM), thrombin (0.1 U/mL), and AA (900 μM) in rat platelets. However, with the exception of the aforementioned, no other study has reported on platelet activation, especially in humans. This study is the first report to state that metformin (1.2–1.8 mM) suppressed collagen-induced (1 μg/mL) platelet aggregation in washed human platelets in a concentration-dependent manner (Figure 1A); however, metformin had only a slight or nonsignificant effect under stimulation by AA (60 μM), U46619 (1 μM; a prostaglandin endoperoxide analogue compound), and thrombin (0.05 U/mL; Figures 1B–D and S1). In addition, metformin at 2–4 mM also exhibited a concentration-dependent inhibitory effect in platelet aggregation stimulated by thrombin (Figure S2). These results showed that the effectiveness of metformin in human platelets differed from that of other agonists. Therefore, collagen was used as an ideal agonist to unravel the inhibitory mechanisms of metformin in human platelet activation. In addition, the LDH assay revealed that metformin (2–10 mM) did not change the release of LDH when it pretreated with platelets for 20 min, which indicates that metformin did not have a cytotoxic effect on the platelets (Figure 1E).

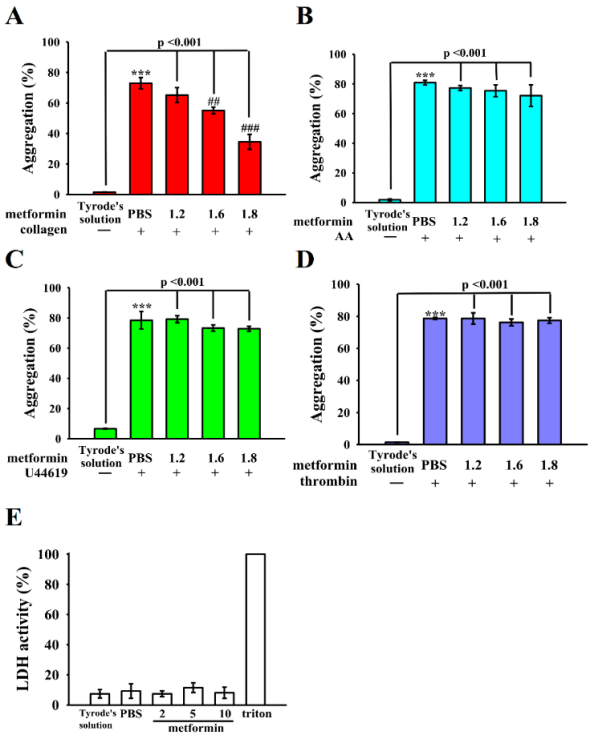

Figure 1. Effect of metformin on platelet aggregation upon stimulation with agonists and lactate dehydrogenase (LDH) release. Washed human platelets (3.6×10^8 cells/mL) were preincubated with a solvent control (PBS) or metformin (1.2, 1.6, and 1.8 mM) and treated with collagen ((**A**), 1 μg/mL), arachidonic acid (AA; (**B**), 60 μM), U46619 (**C**), 1 μM), and thrombin ((**D**), 0.05 U/mL) to trigger platelet aggregation. (**E**) To evaluate the cytotoxic effect of the metformin on the human platelets, the platelets were pretreated with solvent control (PBS) or metformin (2, 5, and 10 mM) or the positive control (0.5% Triton) for 20 min, and 10 μL of the supernatant was dropped on a Fuji Dri-Chem slide LDH-PIII. Data are presented as mean ± standard error of the mean ($n = 4$). *** $p < 0.001$ vs. the resting group (Tyrode's solution); ## $p < 0.01$ and ### $p < 0.001$ vs. PBS + collagen group.

3.2. Effects of Metformin on ATP Release, Relative [Ca^{2+}]i Mobilization, and Surface P-Selectin Expression

Several lines of evidence have indicated that platelet activation is directly associated with the release of granular contents, such as ATP and Ca^{2+}. This release results in an upsurge in platelet aggregation, which will result in strong platelet activation. As presented in Figure 2A,B, metformin significantly suppressed both the ATP release reaction and [Ca^{2+}]i mobilization stimulated by collagen. The relative data are expressed as inhibition percentages on the right-hand side of each figure (A, ATP release reaction: metformin 1.2 mM, 81.1 ± 5.5%; 1.8 mM, 80.7 ± 9.8%, n = 4; B, [Ca^{2+}]i mobilization: metformin 1.2 mM, 84.1 ± 4.1%; 1.8 mM, 37.8 ± 11.0%, n = 4). Furthermore, P-selectin is a key biomarker for platelet activation. For instance, P-selectin is expressed on the inner face of α-granules in a normal condition; however, when platelets become activated, they expose the inner face of the granules to the outer parts of the cells [18]. As indicated in Figure 2C, metformin significantly decreased the collagen-stimulated FITC–P-selectin expression in the flow cytometry study; the results are presented in the right panel of Figure 2C (a, resting control (Tyrode's solution), 95.7 ± 14.5; b, collagen-activated platelets, 262.3 ± 14.1; c, metformin 1.2 mM, 221.3 ± 16.8; d, 1.8 mM, 198.7 ± 7.2; n = 4).

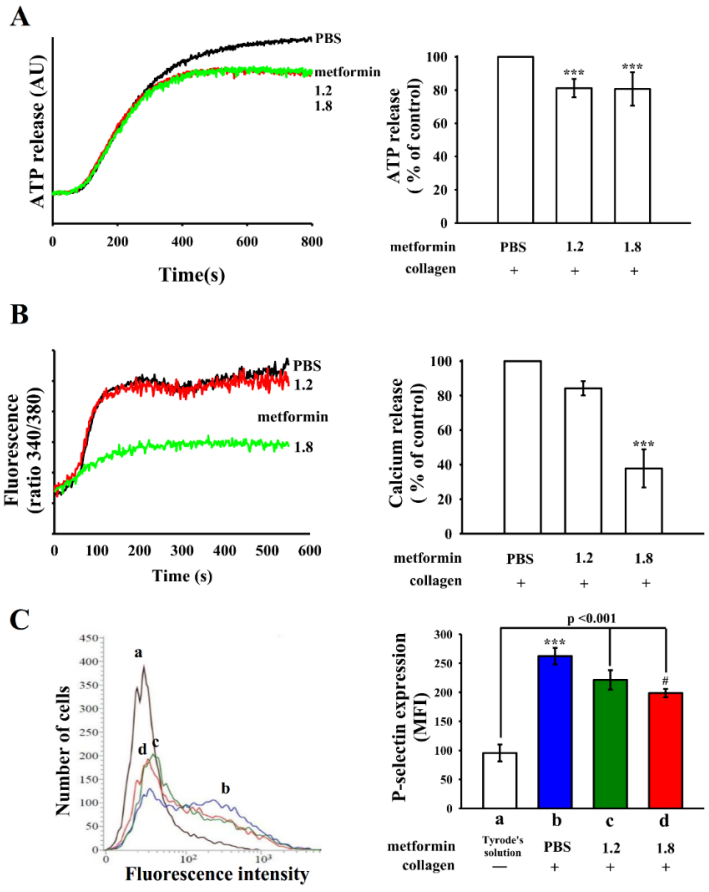

Figure 2. Inhibitory activity of metformin in ATP release, relative [Ca^{2+}]i mobilization, and surface P-selectin expression in human platelets. Washed platelets (3.6×10^8 cells/mL) were preincubated with PBS (a solvent control) or metformin (1.2 and 1.8 mM) and treated with collagen (1 μg/mL) to stimulate (**A**) ATP release (AU: arbitrary unit), (**B**) relative [Ca^{2+}]i mobilization, and (**C**) surface P-selectin expression (MFI: mean fluorescence intensity) (a, Tyrode's solution; b, collagen-activated; c, metformin 1.2 mM; and d, metformin 1.8 mM); each method is detailed in the Section 2. The respective statistical analyses are shown in the bar diagrams. Results are expressed as mean ± standard error of the mean (n = 4). *** $p < 0.001$ vs. PBS + collagen group (**A**,**B**) or Tyrode's solution (**C**); # $p < 0.05$ vs. PBS + collagen group (**C**).

3.3. Influence of Metformin in PLCγ2-Mediated Downstream Signals

Collagen receptor glycoprotein VI (GPVI) forms a complex with the Fc receptor γ-chain (FcRγ) containing immunoreceptor tyrosine-based activation motifs (ITAM) and is phosphorylated by Src-family kinases, such as Fyn or Lyn [19]. In platelets, cross-linking of the GPVI/FcRγ complex would enable the GPVI-bound Fyn or Lyn to move to a position close enough to FcRγ that it would catalyze the phosphorylation of FcRγ ITAM. In turn, this triggers the phosphorylation of downstream signals, leading to the activation of a kinase cascade (i.e., PLCγ2). PLC, a phosphatase, hydrolyzes phosphatidylinositol 4, 5-bisphosphate, which yields two key secondary messengers, diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG activates PKC, triggering 47-kDa protein phosphorylation (pleckstrin or p47). This triggers granule secretion, which results in an increased ATP release; the main role of IP_3 is to elevate calcium influx [16]. Metformin decreased significantly PLCγ2 phosphorylation and PKC activation (p-p47) in collagen-activated platelets (Figures 3A and 4A). In addition, confocal microscopy revealed that metformin (1.8 mM) reduced the phosphorylation of both PLCγ2 and PKC (green fluorescence) in activated platelets (Figures 3B and 4B). However, metformin did not significantly reduce phorbol 12,13-dibutyrate (PDBu; PKC activator)-induced platelet aggregation (Figure 4C), indicating that metformin did not directly affect PKC activation. These results suggest that metformin blocks PLCγ2-mediated downstream signaling (i.e., PKC).

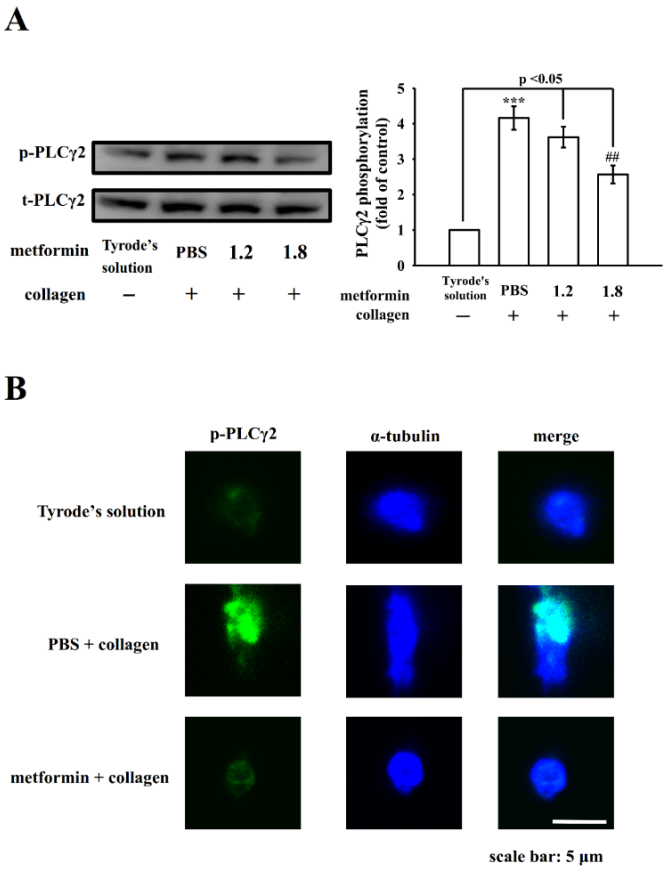

Figure 3. Effectiveness of metformin in phospholipase Cγ2 activation in platelets. Washed platelets were preincubated with PBS or metformin (1.2 and 1.8 mM) and treated with collagen (1 μg/mL) for immunoblotting of (**A**) phospholipase Cγ2 (PLCγ2) activation or (**B**) detecting confocal images of phosphorylated PLCγ2 (green fluorescence) and α-tubulin (blue fluorescence) using goat anti-rabbit CF^{TM} 488A and anti-mouse CF^{TM} 405M Dyes, respectively. Data are presented as mean ± standard error of the mean (n = 4). *** $p < 0.001$ vs. Tyrode's solution group; ## $p < 0.01$ vs. PBS + collagen group. The confocal images are the representative of four independent experiments. Bar: 5 μm.

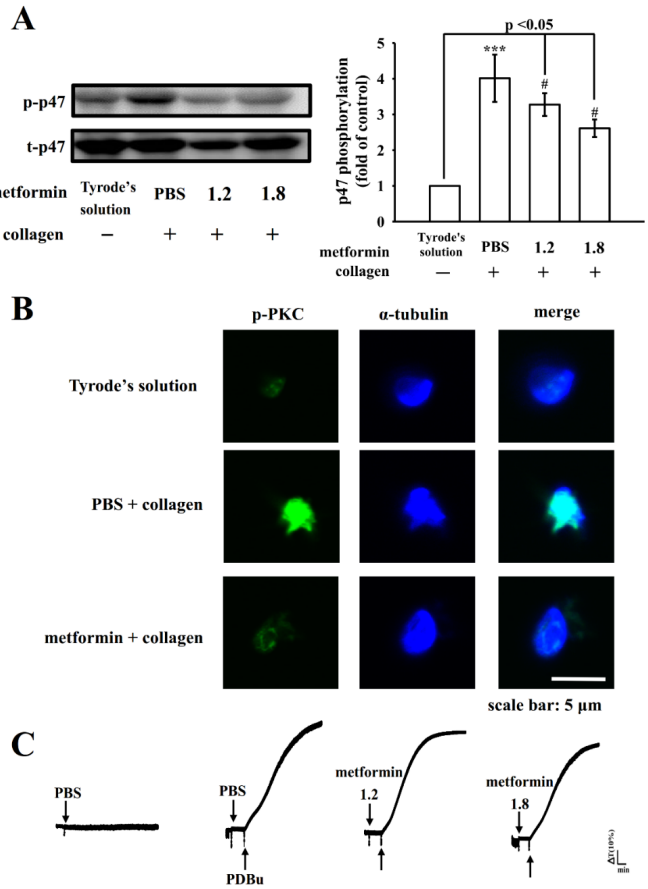

Figure 4. Effectiveness of metformin in protein kinase C activation in platelets. Washed platelets were preincubated with PBS or metformin (1.2 and 1.8 mM) and treated with collagen (1 μg/mL) for immunoblotting of (**A**) protein kinase C (PKC) activation (pleckstrin; p-p47) or (**B**) detecting confocal images of PKC activation (green fluorescence) and α-tubulin (blue fluorescence) using goat anti-rabbit CF^{TM} 488A and anti-mouse CF^{TM} 405M Dyes, respectively. (**C**) For other study, washed platelets were preincubated with PBS or metformin (1.2 and 1.8 mM) and treated with phorbol 12,13-dibutyrate (PDBu, 150 nM) to stimulate platelet aggregation. Data are presented as mean ± standard error of the mean (n = 4). *** $p < 0.001$ vs. Tyrode's solution group; # $p < 0.05$ vs. PBS + collagen group. The confocal images and profiles in (**B**) are the representative of four independent experiments. Bar: 5 μm.

3.4. Regulatory Activity of PI3K–Akt–GSK3β and MAPK Activation by Metformin

A study indicated that high shear stress induces thrombus formation via PI3K/Akt/GSK3β [20]. PI3K stimulation is greatly involved in platelet activation and plays a critical role in regulating Akt activation [20]. Akt (protein kinase B (Ser/Thr kinase)) is found to be stimulated by several platelet agonists; in platelets, GSK3β, a classical enzyme, is controlled downstream of the PI3K/Akt pathway [21]. In the present study, metformin at concentrations of 1.2 and 1.8 mM markedly reduced the phosphorylation of the PI3K/Akt/GSK3β pathway under collagen stimulation (Figure 5). The corresponding data are presented on the right-hand side of each figure. MAPK signals are involved in many cellular activities, such as inflammation, cell proliferation, apoptosis, and platelet activation; the MAPKs that are most often observed in platelets are ERK1/2, JNK1/2, and p38 MAPK [22]. Metformin (1.2 and 1.8 mM) reduced p38 MAPK but not ERK1/2 and JNK1/2 phosphorylation, indicating that p38 MAPK molecule plays a vital role in the metformin-mediated antiplatelet effects (Figure 6). These results indicate that inhibition of PI3K/Akt/GSK3β and p38 MAPK activation may be critical contributors to the antiplatelet effects of metformin.

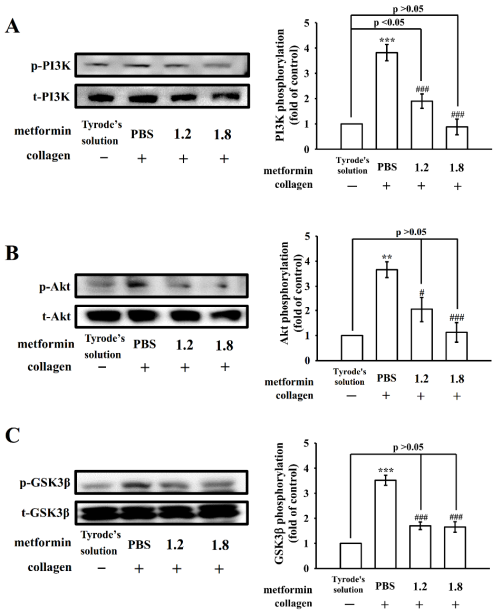

Figure 5. Regulatory effect of metformin in phosphoinositide 3-kinase/Akt/glycogen synthase kinase-3β. Washed platelets were preincubated with PBS or metformin (1.2 and 1.8 mM) and treated with collagen (1 μg/mL) for immunoblotting of (**A**) phosphoinositide 3-kinase (PI3K), (**B**) Akt, and (**C**) glycogen synthase kinase-3β (GSK3β) phosphorylation. Results are expressed as mean ± standard error of the mean (n = 4). ** $p < 0.01$ and *** $p < 0.001$ vs. Tyrode's solution; # $p < 0.05$ and ### $p < 0.001$ vs. PBS + collagen group.

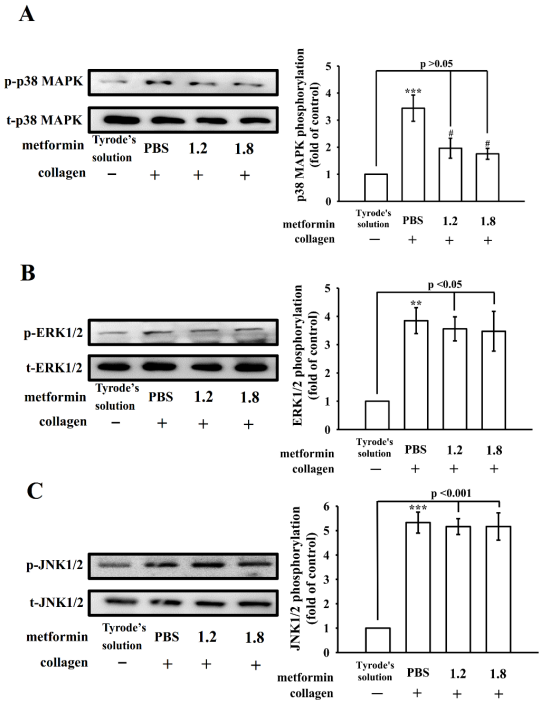

Figure 6. Regulatory activity of metformin in mitogen-activated protein kinase phosphorylation in platelets. Washed platelets were preincubated with PBS or metformin (1.2 and 1.8 mM) and treated with collagen (1 μg/mL) for immunoblotting of (**A**) p38 mitogen-activated protein kinase (p38 MAPK), (**B**) ERK1/2, and (**C**) JNK1/2 phosphorylation. Data are expressed as mean ± standard error of the mean (n = 4). ** $p < 0.01$ and *** $p < 0.001$ vs. Tyrode's solution; # $p < 0.05$ vs. PBS + collagen group.

3.5. Effect of Metformin in Phosphorylation of VASP

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are small cyclic monophosphates. These nucleotides are vital messengers in several signaling pathways involved in the regulation of multiple targets, especially in the phosphorylation of VASP. As presented in Figure 7, PGE_1 (20 nM) and NTG (10 μM) triggered $VASP^{ser157}$ and $VASP^{ser239}$ phosphorylation, respectively; treatment with H89 (a protein kinase A inhibitor; 100 μM) and KT5823 (a protein kinase G inhibitor; 10 μM) reduced $VASP^{ser157}$ and $VASP^{Ser239}$ phosphorylation, respectively. However, metformin (1.8 mM) had no significant effects on either type of VASP phosphorylation, indicating that cyclic nucleotides may not participate in metformin-mediated antiplatelet activation.

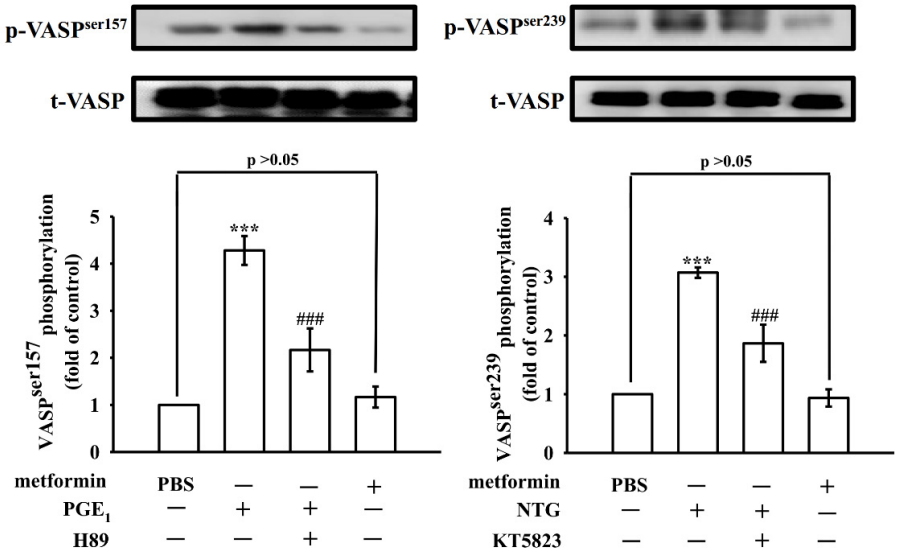

Figure 7. Influence of metformin in vasodilator-stimulated phosphoprotein (VASP) phosphorylation in human platelets. Washed platelets (3.6×10^8 cells/mL) were directly stimulated by prostaglandin E_1 (PGE_1, 20 nM), nitroglycerin (NTG, 10 μM), or metformin (1.8 mM) in the presence of H89 (100 μM) or KT5823 (10 μM) for immunoblotting of VASP phosphorylation. Results are expressed as mean ± standard error of the mean (n = 4). *** $p < 0.001$ vs. PBS-treated group; ### $p < 0.001$ vs. PGE_1- or NTG-treated group.

3.6. Effect of Metformin on Acute Pulmonary Thrombosis and Bleeding Time in Mice

The antithrombotic therapeutic effect of metformin in mice was also evaluated with respect to preventing mortality by the effect of acute pulmonary thromboembolism (Figure 8A). Metformin at 250 mg/kg, but not 150 mg/kg, significantly reduced the ADP (0.7 mg/g)-induced mortality rate from 100% (12 dead, n = 12; PBS) to 83.3% (10 dead, n = 12; metformin 150 mg/kg) and 50% (6 dead, n = 12; metformin 250 mg/kg). All the animals were alive in the sham group (injected PBS without ADP; n = 12). However, heparin (1.5 U/g) had no effects (11 dead, n = 12). We also evaluated the bleeding time through transection of the mouse tails 30 min after metformin was intraperitoneally administered; the bleeding times were 204 ± 50 s (PBS-treated group; n = 8), 228 ± 27 s (150 mg/kg metformin-treated group; n = 8), 295 ± 81 s (250 mg/kg metformin-treated group; n = 8), and 526 ± 33 s (1.5 U/g heparin-treated group; n = 8; Figure 8B). To detect if there are any rebleeding occurrences, each mouse was separately watched for 15 min, even after the bleeding had stopped. The results suggest that metformin reduced acute pulmonary thrombosis without significantly prolonging bleeding time. These findings provide a possibility that metformin may be employed in the protection of cardiovascular events and reduction in mortality in patients with diabetes [23].

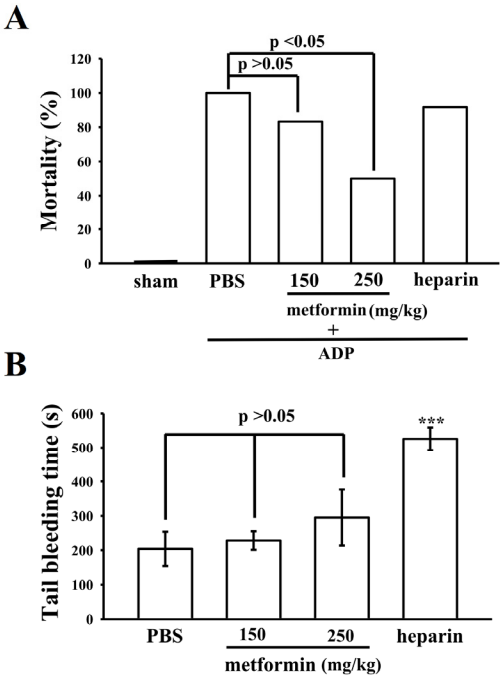

Figure 8. Influence of metformin in acute pulmonary thromboembolism and tail bleeding time in vivo. (**A**) For acute pulmonary thrombosis, PBS or metformin (150 and 250 mg/kg) or heparin (1.5 U/g) was intraperitoneally administered to mice, and ADP (0.7 mg/g) was injected into the tail veins. (**B**) The bleeding time was measured through mouse tail transection after 30 min of intraperitoneal administration of either PBS or metformin (150 and 250 mg/kg) or heparin (1.5 U/g). Results are expressed as mean ± standard error of the mean ((**A**), $n = 12$; (B), $n = 8$). *** $p < 0.001$ vs. PBS-treated group. Data in (**A**) are presented as the percentage of mortality.

4. Discussion

Thrombotic cardiovascular diseases cause 65% death in diabetic patients. Research has demonstrated that 10 to 40% of patients with diabetes are biochemically resistant to aspirin treatment. Therefore, a clearer understanding of existing new therapies targeting the underlying mechanisms on platelet activation must be established [1,2]. Studies have reported that metformin is a potential drug candidate as it is associated with a decreased rate of mortality in diabetes-associated CVDs and cerebrovascular diseases in those with T2D [23–25]. The findings of this study reveal that metformin possesses significant antiplatelet activity against collagen-stimulated human platelets, in addition to its other well-known properties.

In this study, metformin was only effective on inhibiting platelet aggregation induced by collagen but it exerted a very slight or even no effect on other agonist (i.e., AA, U46619, and thrombin)-induced platelet activation. Platelet activation is triggered by different physiological agonists, which show their effects through interactions with specific receptors on platelet membranes. Among these platelet agonists, collagen plays a key role in platelet activation. Platelets adhere to the connective tissue protein in collagen, resulting in a change in the shape and release of granules. Adhesion is partly dependent on $[Ca^{2+}]i$ mobilization, ADP/ATP release, and thromboxane A_2 formation, and aggregation is entirely dependent on this release [7]. Collagen is present in vascular subendothelium and vessel walls, acting as a substrate for platelet adhesion; it is also an endogenous strong platelet activator. Metformin decreased collagen-induced platelet aggregation, which suggests that metformin affects a unique PLC-dependent mechanism. PLC activation leads to IP_3 and DAG formation, which, in turn, activates PKC and induces p47 protein phosphorylation [26]. The PLCγ family comprises isozymes 1 and 2, and PLCγ2 contributes in collagen-dependent signaling in platelets [27]. The PKC family plays a vital role in serine/threonine kinase phosphorylation, which considerably affects numerous signal transduction pathways in

most cells [28]. This study found that metformin reduced collagen-induced PLCγ2/PKC activation. However, it did not directly affect PKC activation because PDBu-induced platelet aggregation was not reduced, suggesting that PLCγ2-mediated signaling plays a vital role in metformin antiplatelet activity. Platelet activation is usually associated with an increase in $[Ca^{2+}]$i mobilization and ATP release, as well as surface P-selectin expression due to granule secretion; α-granules are mostly located in platelet protein storage compartments and contain P-selectin, membrane-associated proteins and fibrinogen, and platelet-derived growth factor, soluble proteins. In platelet activation, α-granules exocytosis represents as a marker and, therefore, we evaluated α-granule exocytosis via flow cytometric analysis of P-selectin expression (Figure 2C).

Almost all the isoforms of PI3K (i.e., α, β, γ, and δ) expressed in platelet contribute extensively in platelet activation. PI3K isoforms are crucial downstream actors of many platelet receptors, such as GPVI (collagen receptor) and the P_2Y_{12} (ADP receptors) and integrin $\alpha_{IIb}\beta_3$ [29]. PI3Kβ is a downstream regulator of the GPVI in terms of the control of PLCγ2 phosphorylation and Ca^{2+} mobilization [30]. Therefore, PI3Kβ may be a key target in preventing arterial thrombosis. Akt is an important effector of PI3K, and its three isoforms (Akt1, Akt2, and Akt3) are ubiquitously expressed in human platelets [20]. Akt-knockout mice reportedly showed platelet activation defects [31]. Therefore, the contribution of protein kinases, particularly PI3Kβ, upon Akt activation, may establish as targets for antithrombotic therapy. Although the downstream regulators of Akt that participate in platelet activation remain unknown, several candidates of GSK3, such as α and β isoforms, have been identified [32]. Both the α and β isoforms are expressed in platelets, although GSK3β is the most abundant [33]. GSK3 inhibition may be required for full platelet response under stimulation by some agonists. Platelet-specific PI3Kβ knockout mice showed arterial thrombus instability under high shear stress due to diminished Akt activation and GSK3 inhibition [20]. Nevertheless, the mechanisms underlying GSK3 regulation of platelet activation remain unclear. Therefore, identifying the platelet substrates of GSK3 may enable identification of new antithrombotic candidates. In summary, PI3K/Akt/GSK3β signaling plays a dominant role in platelet activation and arterial thrombus growth in vivo.

MAPKs are serine/threonine kinases that exchange extracellular stimuli into a wide range of cellular responses. MAPKs comprise three major subgroups, such as ERK1/2, p38 MAPK, and JNK1/2. ERK1/2 isoform is reported to involve in proliferation, adhesion, and cell progression [34]. p38 MAPK and JNK1/2 are involved in apoptosis [34]. MAPK-specific inhibitors and their knockout in mice demonstrate that ERK1/2, JNK1/2, and p38 MAPK are involved in platelet activation [35]. Although the mechanisms of JNK1/2 and ERK1/2 in platelets remain unclear, research demonstrates that the inhibition of integrin $\alpha_{IIb}\beta_3$ activation may be involved [36]. In addition, p38 MAPK provides a crucial signal in aggregation caused by collagen [37]. This may explain why metformin exhibits more potent activity in reducing collagen-stimulated platelet activation than other agonists do. Of the several reported downstream targets of p38 MAPK, the most physiologically relevant in platelets is cytosolic phospholipase A_2, which is stimulated by various platelet agonists to catalyze AA release to produce thromboxane A_2 [38]. Moreover, both PI3K/Akt and MAPKs are equally activated in platelets, while PKC acts as an upstream regulator of these molecules [31].

In this study, metformin (1.8 mM) exerted no significant effects on $VASP^{ser157}$ and $VASP^{ser239}$ phosphorylation in human platelets. Elevation of intracellular cAMP and cGMP in platelets activates their individual protein kinase A and protein kinase G, which control platelet activation through phosphorylation of intracellular protein substrates, namely VASP (Ser^{157}) and (Ser^{239}), respectively [39]. Cyclic nucleotide elevation decreases the Ca^{2+} influx and reduces the activity of cell membrane-bound calcium transporters, which suppresses PLC/PKC signaling activation. Ke et al. [40] reported that metformin stimulates protein kinase A activation in human umbilical vein endothelial cell; however, this study eliminates the possibility of cyclic nucleotide/VASP signal involvement in metformin-mediated antiplatelet activity.

Antiplatelet therapy has reportedly been effective in reducing the mortality and morbidity of CVDs. However, the antiplatelet drugs prescribed in clinical practice create serious side effects, including bleeding, gastrointestinal toxicity, neutropenia, and thrombocytopenia. A noted disadvantage on using current antiplatelet therapies is their inability to separate the bleeding side effect [41,42]. The influence of metformin on hemostatic function was demonstrated in our tail bleeding time experiment. We observed that metformin prevents acute pulmonary thromboembolism in mice without a significantly prolonged bleeding time. In addition, aspirin (1 mg/kg)-treated mice obviously prolonged bleeding time (data not shown). Aspirin is one of the most well-known and widely available antiplatelet therapies used in both primary and secondary prevention of CVDs; however, it has the limitation of undesirable extension of bleeding time. Animal models of microvascular thrombosis are essential in understanding the effectiveness of test compounds for treating this condition. Our study advocates the necessity for further investigation of metformin, as it could lead novel antiplatelet drug development strategy with fewer side effects.

5. Conclusions

Our results provide new insights into the role and mechanisms of metformin in human platelet activation. Metformin efficiently diminished platelet activation by interfering with the PLCγ2/PKC, PI3K/Akt/GSK3β, and p38 MAPK signaling pathways through a cyclic nucleotide/VASP-independent mechanism (Figure 9). The present results highlight a novel therapeutic drug for platelet abnormalities in diabetes mellitus and a general strategy for preventing cardiovascular complications. Metformin could be a new class of antiplatelet agent that is effective at inhibiting platelet activation.

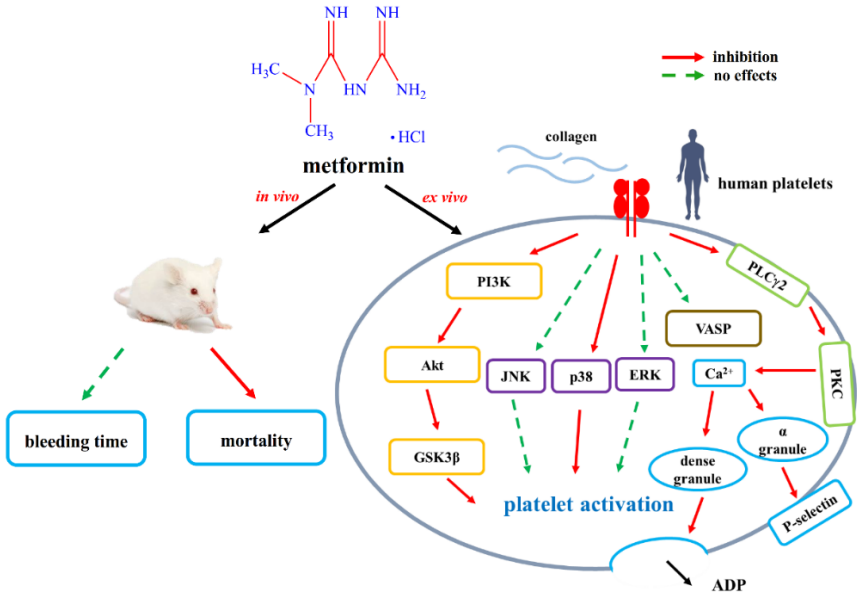

Figure 9. Summary scheme graphic showing suppressive signaling by metformin in human platelet activation, acute pulmonary thromboembolism, and tail bleeding time in mice. Collagen binds to its receptors, and then stimulates PLCγ2-PKC cascade, PI3K-Akt-GSK3β, and p38 MAPK, followed by the suppression of platelet activation, ultimately reducing the death rate associated with acute pulmonary thromboembolism without increasing the bleeding time.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12157426/s1, Figure S1: Inhibitory curves of metformin in platelet aggregation stimulated by various agonists. Washed human platelets (3.6×10^8 cells/mL) were preincubated with a solvent control (PBS, black line) or metformin (1.2 mM, red line; 1.6 mM, green line; 1.8 mM, blue line) and subsequently treated with (A) collagen (1 μg/mL), (B) thrombin (0.05 U/mL), (C) U46619 (1 μM), or (D) arachidonic acid (AA; 60 μM) to stimulate platelet aggregation. The data are the representative of four independent experiments.; Figure S2: Inhibitory activity of metformin in thrombin-induced platelet aggregation. Washed human platelets (3.6×10^8 cells/mL) were preincubated with a solvent control (PBS) or metformin (2–4 mM) and subsequently treated with thrombin (0.05 U/mL) to stimulate platelet aggregation. The corresponding statistical data is displayed in the below panel. Data are expressed as mean ± standard error of the mean (n = 4). *** $p < 0.001$ vs. Tyrode's solution; ### $p < 0.001$ vs. PBS + thrombin group.

Author Contributions: Y.C. performed the research and drafted the paper; W.-C.H., C.-Y.H. (Chia-Yuan Hsu), and C.-W.H. performed the research and contributed to the experiments; T.J. and C.-Y.H. (Cheng-Ying Hsieh). contributed to the investigation and analyzed the data; W.-J.L. and C.-C.C. perceived and planned the study. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was approved by the Institutional Review Board of Taipei Medical University (TMU-JIRB-N201812024) and conformed to the directives of the Declaration of Helsinki. All animal experiments and care procedures conformed to the Guide for the Care and Use of Laboratory Animals (LAC-2020-0294) and were approved by the Institutional Animal Care and Use Committee of Taipei Medical University.

Informed Consent Statement: Informed consent was obtained from all study participants.

Data Availability Statement: All data generated or analyzed in this study are included in this article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Khan, M.A.B.; Hashim, M.J.; King, J.K.; Govender, R.D.; Mustafa, H.; Al Kaabi, J. Epidemiology of Type 2 Diabetes—Global Burden of Disease and Forecasted Trends. *J. Epidemiol. Glob. Health* **2020**, *10*, 107–111. [CrossRef]
2. Bhutani, J.; Bhutani, S. Worldwide Burden of Diabetes. *Indian. J. Endocrinol. Metab.* **2014**, *18*, 868–870. [CrossRef]
3. Bai, B.; Chen, H. Metformin: A Novel Weapon Against Inflammation. *Front. Pharmacol.* **2021**, *12*, 622262. [CrossRef] [PubMed]
4. Shin, J.I.; Sang, Y.; Chang, A.R.; Dunning, S.C.; Coresh, J.; Inker, L.A.; Selvin, E.; Ballew, S.H.; Grams, M.E. The FDA Metformin Label Change and Racial and Sex Disparities in Metformin Prescription Among Patients with CKD. *J. Am. Soc. Nephrol.* **2020**, *31*, 1847–1858. [CrossRef] [PubMed]
5. Ala, M.; Ala, M. Metformin for Cardiovascular Protection, Inflammatory Bowel Disease, Osteoporosis, Periodontitis, Polycystic Ovarian Syndrome, Neurodegeneration, Cancer, Inflammation and Senescence: What Is Next? *ACS Pharmacol. Transl. Sci.* **2021**, *4*, 1747–1770. [CrossRef] [PubMed]
6. Sheu, J.R.; Yen, M.H.; Hung, W.C.; Lee, Y.M.; Su, C.H.; Huang, T.F. Triflavin Inhibits Platelet-Induced Vasoconstriction in de-Endothelialized Aorta. *Arterioscler. Thromb. Vasc. Biol.* **1997**, *17*, 3461–3468. [CrossRef]
7. Stegner, D.; Nieswandt, B. Platelet Receptor Signaling in Thrombus Formation. *J. Mol. Med.* **2011**, *89*, 109–121. [CrossRef]
8. Salvatore, T.; Pafundi, P.C.; Galiero, R.; Rinaldi, L.; Caturano, A.; Vetrano, E.; Aprea, C.; Albanese, G.; Di Martino, A.; Ricozzi, C.; et al. Can Metformin Exert as an Active Drug on Endothelial Dysfunction in Diabetic Subjects? *Biomedicines* **2020**, *9*, 3. [CrossRef]
9. Anfossi, G.; Russo, I.; Bonomo, K.; Trovati, M. The Cardiovascular Effects of Metformin: Further Reasons to Consider an Old Drug as a Cornerstone in the Therapy of Type 2 Diabetes Mellitus. *Curr. Vasc. Pharmacol.* **2010**, *8*, 327–337. [CrossRef]
10. Xin, G.; Wei, Z.; Ji, C.; Zheng, H.; Gu, J.; Ma, L.; Huang, W.; Morris-Natschke, S.L.; Yeh, J.L.; Zhang, R.; et al. Metformin Uniquely Prevents Thrombosis by Inhibiting Platelet Activation and mtDNA Release. *Sci. Rep.* **2016**, *6*, 36222. [CrossRef]
11. Gin, H.; Freyburger, G.; Boisseau, M.; Aubertin, J. Study of the Effect of Metformin on Platelet Aggregation in Insulin-Dependent Diabetics. *Diabetes Res. Clin. Pract.* **1989**, *6*, 61–67. [CrossRef]

12. De Caterina, R.; Marchetti, P.; Bernini, W.; Giannarelli, R.; Giannessi, D.; Navalesi, R. The Direct Effects of Metformin on Platelet Function In Vitro. *Eur. J. Clin. Pharmacol.* **1989**, *37*, 211–213. [CrossRef]
13. Gargiulo, P.; Caccese, D.; Pignatelli, P.; Brufani, C.; De Vito, F.; Marino, R.; Lauro, R.; Violi, F.; Di Mario, U.; Sanguigni, V. Metformin Decreases Platelet Superoxide Anion Production in Diabetic Patients. *Diabetes Metab. Res. Rev.* **2002**, *18*, 156–159. [CrossRef]
14. Formoso, G.; De Filippis, E.A.; Michetti, N.; Di Fulvio, P.; Pandolfi, A.; Bucciarelli, T.; Ciabattoni, G.; Nicolucci, A.; Davì, G.; Consoli, A. Decreased In Vivo Oxidative Stress and Decreased Platelet Activation Following Metformin Treatment in Newly Diagnosed Type 2 Diabetic Subjects. *Diabetes Metab. Res. Rev.* **2008**, *24*, 231–237. [CrossRef]
15. Chen, W.F.; Lee, J.J.; Chang, C.C.; Lin, K.H.; Wang, S.H.; Sheu, J.R. Platelet Protease-Activated Receptor (PAR)4, but not PAR1, Associated with Neutral Sphingomyelinase Responsible for Thrombin-Stimulated Ceramide-NF-κB Signaling in Human Platelets. *Haematologica* **2013**, *98*, 793–801. [CrossRef]
16. Sheu, J.R.; Lee, C.R.; Lin, C.H.; Hsiao, G.; Ko, W.C.; Chen, Y.C.; Yen, M.H. Mechanisms Involved in the Antiplatelet Activity of Staphylococcus Aureus Lipoteichoic Acid in Human Platelets. *Thromb. Haemost.* **2000**, *83*, 777–784. [CrossRef]
17. Sheu, J.R.; Hung, W.C.; Wu, C.H.; Lee, Y.M.; Yen, M.H. Antithrombotic Effect of *Rutaecarpine*, an Alkaloid Isolated from *Evodia Rutaecarpa*, on Platelet Plug Formation in vivo Experiments. *Br. J. Haematol.* **2000**, *110*, 110–115. [CrossRef]
18. Merten, M.; Thiagarajan, P. P-selectin expression on platelets determines size and stability of platelet aggregates. *Circulation* **2000**, *102*, 1931–1936. [CrossRef]
19. Moroi, M.; Jung, S.M. Platelet Glycoprotein VI: Its Structure and Function. *Thromb. Res.* **2004**, *114*, 221–233. [CrossRef]
20. Laurent, P.A.; Severin, S.; Gratacap, M.P.; Payrastre, B. Class I PI 3-Kinases Signaling in Platelet Activation and Thrombosis: PDK1/Akt/GSK3 Axis and Impact of PTEN and SHIP1. *Adv. Biol. Regul.* **2014**, *54*, 162–174. [CrossRef]
21. Manning, B.D.; Cantley, L.C. AKT/PKB Signaling: Navigating Downstream. *Cell* **2007**, *129*, 1261–1274. [CrossRef]
22. Cargnello, M.; Roux, P.P. Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. *Microbiol. Mol. Biol. Rev.* **2011**, *75*, 50–83. [CrossRef]
23. Inzucchi, S.E. Metformin and Heart Failure: Innocent Until Proven Guilty. *Diabetes Care* **2005**, *28*, 2585–2587. [CrossRef]
24. Roussel, R.; Travert, F.; Pasquet, B.; Wilson, P.W.; Smith, S.C., Jr.; Goto, S.; Ravaud, P.; Marre, M.; Porath, A.; Bhatt, D.L.; et al. Reduction of Atherothrombosis for Continued Health (REACH) Registry Investigators. Metformin Use and Mortality Among Patients with Diabetes and Atherothrombosis. *Arch. Intern. Med.* **2010**, *170*, 1892–1899. [CrossRef]
25. Lu, D.Y.; Huang, C.C.; Huang, P.H.; Chung, C.M.; Lin, S.J.; Chen, J.W.; Chan, W.L.; Leu, H.B. Metformin Use in Patients with Type 2 Diabetes Mellitus is Associated with Reduced Risk of Deep Vein Thrombosis: A Non-Randomized, Pair-Matched Cohort Study. *BMC Cardiovasc. Disord.* **2014**, *14*, 187. [CrossRef]
26. Liu, Y.; Liu, T.; Ding, K.; Liu, Z.; Li, Y.; He, T.; Zhang, W.; Fan, Y.; Ma, W.; Cui, L.; et al. Phospholipase Cγ2 Signaling Cascade Contribute to the Antiplatelet Effect of Notoginsenoside Fc. *Front. Pharmacol.* **2018**, *9*, 1293. [CrossRef]
27. Ragab, A.; Séverin, S.; Gratacap, M.P.; Aguado, E.; Malissen, M.; Jandrot-Perrus, M.; Malissen, B.; Ragab-Thomas, J.; Payrastre, B. Roles of the C-Terminal Tyrosine Residues of LAT in GPVI-Induced Platelet Activation: Insights into the Mechanism of PLC Gamma 2 Activation. *Blood* **2007**, *110*, 2466–2474. [CrossRef]
28. Varga-Szabo, D.; Braun, A.; Nieswandt, B. Calcium Signaling in Platelets. *J. Thromb. Haemost.* **2009**, *7*, 1057–1066. [CrossRef]
29. Gratacap, M.P.; Guillermet-Guibert, J.; Martin, V.; Chicanne, G.; Tronchère, H.; Gaits-Iacovoni, F.; Payrastre, B. Regulation and Roles of PI3Kβ, a Major Actor in Platelet Signaling and Functions. *Adv. Enzyme Regul.* **2011**, *51*, 106–116. [CrossRef]
30. Jackson, S.P.; Schoenwaelder, S.M.; Goncalves, I.; Nesbitt, W.S.; Yap, C.L.; Wright, C.E.; Kenche, V.; Anderson, K.E.; Dopheide, S.M.; Yuan, Y.; et al. PI 3-Kinase p110Beta: A New Target for Antithrombotic Therapy. *Nat. Med.* **2005**, *11*, 507–514. [CrossRef] [PubMed]
31. Jayakumar, T.; Chen, W.F.; Lu, W.J.; Chou, D.S.; Hsiao, G.; Hsu, C.Y.; Sheu, J.R.; Hsieh, C.Y. A Novel Antithrombotic Effect of Sulforaphane via Activation of Platelet Adenylate cyclase: Ex vivo and in vivo Studies. *J. Nutr. Biochem.* **2013**, *24*, 1086–1095. [CrossRef] [PubMed]
32. Woulfe, D.S. Akt Signaling in Platelets and Thrombosis. *Expert Rev. Hematol.* **2010**, *2010 3*, 81–91. [CrossRef]
33. Li, D.; August, S.; Woulfe, D.S. GSK3 Beta is a Negative Regulator of Platelet Function and Thrombosis. *Blood* **2008**, *111*, 3522–3530. [CrossRef] [PubMed]
34. Bugaud, F.; Nadal-Wollbold, F.; Lévy-Toledano, S.; Rosa, J.P.; Bryckaert, M. Regulation of c-jun-NH2 Terminal Kinase and Extracellular-Signal Regulated Kinase in Human Platelets. *Blood* **1999**, *94*, 3800–3805. [CrossRef]
35. Fan, X.; Wang, C.; Shi, P.; Gao, W.; Gu, J.; Geng, Y.; Yang, W.; Wu, N.; Wang, Y.; Xu, Y.; et al. Platelet MEKK3 Regulates Arterial Thrombosis and Myocardial Infarct Expansion in Mice. *Blood Adv.* **2018**, *2*, 1439–1448. [CrossRef]
36. Hughes, P.E.; Renshaw, M.W.; Pfaff, M.; Forsyth, J.; Keivens, V.M.; Schwartz, M.A.; Ginsberg, M.H. Suppression of Integrin Activation: A Novel Function of a Ras/Raf-Initiated MAP Kinase Pathway. *Cell* **1997**, *88*, 521–530. [CrossRef]
37. Saklatvala, J.; Rawlinson, L.; Waller, R.J.; Sarsfield, S.; Lee, J.C.; Morton, L.F.; Barnes, M.J.; Farndale, R.W. Role for p38 Mitogen-Activated Protein Kinase in Platelet Aggregation Caused by Collagen or a Thromboxane Analogue. *J. Biol. Chem.* **1996**, *271*, 6586–6589. [CrossRef]
38. Adam, F.; Kauskot, A.; Rosa, J.P.; Bryckaert, M. Mitogen-Activated Protein Kinases in Hemostasis and Thrombosis. *J. Thromb. Haemost.* **2008**, *6*, 2007–2016. [CrossRef]

39. Benz, P.M.; Laban, H.; Zink, J.; Günther, L.; Walter, U.; Gambaryan, S.; Dib, K. Vasodilator-Stimulated Phosphoprotein (VASP)-Dependent and -Independent Pathways Regulate Thrombin-Induced Activation of Rap1b in Platelets. *Cell Commun. Signal.* **2016**, *14*, 21. [CrossRef]
40. Ke, J.; Liu, Y.; Yang, J.; Lu, R.; Tian, Q.; Hou, W.; Wang, G.; Wei, R.; Hong, T. Synergistic Effects of Metformin with Liraglutide Against Endothelial Dysfunction through GLP-1 Receptor and PKA Signalling Pathway. *Sci. Rep.* **2017**, *7*, 41085. [CrossRef]
41. Musumeci, L.; Kuijpers, M.J.; Gilio, K.; Hego, A.; Théâtre, E.; Maurissen, L.; Vandereyken, M.; Diogo, C.V.; Lecut, C.; Guilmain, W.; et al. Dual-Specificity Phosphatase 3 Deficiency or Inhibition Limits Platelet Activation and Arterial Thrombosis. *Circulation* **2015**, *131*, 656–668. [CrossRef] [PubMed]
42. Dayal, S.; Wilson, K.M.; Motto, D.G.; Miller, F.J., Jr.; Chauhan, A.K.; Lentz, S.R. Hydrogen Peroxide Promotes Aging-Related Platelet Hyperactivation and Thrombosis. *Circulation* **2013**, *127*, 1308–1316. [CrossRef] [PubMed]