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Exploring the Potential of Metformin as a Novel Nutritional Intervention for Mitigating Arterial Thrombotic Risk in Human Subjects

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ABSTRACT

Metformin is extensively prescribed as a first-line medication for the management of type 2 diabetes (T2D), a condition frequently coexisting with kidney disease, cardiovascular diseases (CVDs), and retinopathy. The widespread use of metformin is attributed to its well-established safety profile. Notably, platelets play a crucial role in arterial thrombosis, a significant factor contributing to the development of CVDs and cerebrovascular diseases. Our previous study has demonstrated that metformin may reduce collagen-induced platelet activation in human subjects, indicating a positive impact on platelet function. However, its mechanisms in platelet activation stimulated by other agonists remain not fully understood, especially regarding the effect of metformin on thrombin-induced platelet activation. In this study, metformin inhibited platelet aggregation stimulated by thrombin in a concentration-dependent manner. Metformin suppressed P-selectin expression, $[Ca^{2+}]i$ mobilization, ATP-release, and as well as protein kinase C (PKC), Akt and p38 mitogen-activated protein kinase (MAPK). In conclusion, metformin can potentially function as a nutritional agent for long-term use at a low dose to prevent cardiovascular diseases (CVDs) or other chronic diseases.

Keywords
Arterial thrombosis, Human platelets, Metformin, Thrombin.

Introduction

Platelets, which are blood cells without a nucleus, participate in the normal process of blood clotting and the formation of arterial blood clots, which can lead to various cardiovascular diseases (CVDs) and cerebrovascular diseases. Platelets adhere todamaged blood vessel walls, where they release active substances that initiate the aggregation of platelets when there is disruption to the vessel surface [1]. Various physiological factors (such as collagen, thrombin, ADP) activate platelets by interacting with specific receptors on their membranes. These factors have the potential to enhance platelet activation through two primary mechanisms. Firstly, they facilitate the synthesis of thromboxane A2 (TxA2) from arachidonic acid (AA) or the secretion of ADP from dense granules within the platelets. Upon release, ADP binds to two major purinergic receptors (P2Y1 and P2Y12), which play a crucial role in amplifying plateletactivation triggered by other aggregating factors [2]. Inhibiting platelet activation holds significant importance as it can substantially reduce the risk of life-threatening events,such as vascular death, myocardial infarction, and ischemic stroke, especially in individuals with atherosclerotic vascular diseases. Additionally, individuals with diabetes, especially type 2 diabetes (T2D), are at an elevated risk of developing cardiovascular complications due to hyperglycemia-related factors that can causedamage to blood vessel walls. This damage promotes platelet adhesion and aggregation, further increasing the risk of atherosclerotic events, such as myocardial infarction and ischemic stroke.

Metformin, a well-established first-line medication for T2D, is widely used due to its safety and affordability, making it the most commonly prescribed drug for T2D treatment [3]. As a biguanide derivative, metformin effectively lowers blood glucose levels by regulating energy metabolism, including inhibiting hepatic gluconeogenesis, reducing glucose absorption, and enhancing glucose utilization in peripheral tissues [4]. Extensive research has demonstrated that metformin possesses beneficial effects on inflammation, aging-related diseases, obesity, CVDs, chronic kidney disease, inflammatory bowel disease, cancers, osteoporosis, and periodontitis [4]. Clinical trials have shown the beneficial effects of metformin on cardiovascular diseases. For instance, metformin has been found to reduce mortality and thrombotic complications associated with diabetes, such as endothelial dysfunction, myocardial infarction, acute myocarditis, and chronic heart failure [5-8]. Metformin treatment has been observed to inhibit platelet aggregation in healthy human subjects [9] and patients with Type I diabetes [10]. It also reduces oxidative stress in vivo, leading to decreased platelet activation by increasing plasma levels of vitamins A and E or reducing levels of platelet superoxide anions in patients with type 2 diabetes [11,12]. Our previous study showed that metformin inhibited platelet aggregation stimulated by collagen and moderately inhibits thrombin stimulation [13]. Concurrently, we also found that metformin clearly reduces pulmonary thromboembolism without prolonging bleeding time as compared with heparin [13]. Collectively, metformin shows promise as a therapeutic agent for preventing arterial blood clot formation. Although that study examined the inhibitory effects of metformin, its mechanisms in platelet activation remain not fully understood, especially regarding the effect of metformin on thrombin-induced platelet activation. Therefore, this study aimed to investigate the underlying mechanisms of metformin in thrombin-induced human platelet activation.

Materials and Methods

Chemicals and Reagents

Metformin, luciferin–luciferase, heparin, prostaglandin E1 (PGE1), phenylmethylsulfonyl fluoride, sodium orthovanadate, sodium pyrophosphate, aprotinin, leupeptin, sodium fluoride, 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 pAbs and anti-Akt, anti-p38 mitogen-activated protein kinase (MAPK) monoclonal antibodies (mAbs) were purchased from Cell Signaling (Beverly, MA, USA). Anti-phospho-Akt (Ser^{473}) pAb was purchased from BioVision (Mountain View, CA, USA). 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). The FITC-anti-human CD42P (P-selectin) mAb was obtained from BioLegend (San Diego,CA, USA). Protein assay dye reagent concentrate was supplied by Bio-Rad Laboratories (Hercules, CA, USA), while Fura 2-AM was provided by Molecular Probes (Eugene, OR, USA). The stock solution of metformin (100 mM) was dissolved in phosphate-buffered saline (PBS) and stored at 4°C until use.

Platelet Preparation, Aggregation, and ATP-Release Reaction

After obtaining approval from the Institutional Review Board of Taipei Medical University (TMU-JIRB-N201812024) in accordance with the Declaration of Helsinki guidelines, human platelet suspensions were prepared as described previously [14]. Blood samples were collected from healthy human volunteers aged between 20 to 35 years who had not taken any medication for the 14 days prior to collection. The collected blood was mixed with an acid-citrate-dextrose solution (9:1, v/v) and centrifuged at 120 × *g* for 10 min. The resulting supernatant was supplemented with EDTA (2 mM) and heparin (6.4 U/mL) for 5 min before undergoing another centrifugation at 500 × *g* for 10 min. The platelet pellet obtained was resuspended in 5 mL of Tyrode's solution and kept at 37°C for 10 min. Following another spin at 500 × *g* for 10 min, the washing procedure was repeated. The washed platelets were then suspended in Tyrode's solution containing BSA (3.5 mg/mL). Platelet numbers were counted using a Coulter counter (Beckman Coulter, Miami, FL, USA). The concentration of washed platelet suspensions is approximately 3.6×10^8 –1.2×10^9 cells/mL for following studies. The final Ca^{2+} concentration in the Tyrode's solution was 1 mM.

Following the protocol described by Chen et al. [14], platelet aggregation was assessed using a Lumi-Aggregometer (Payton Associates, Scarborough, ON, Canada). Prior to the addition of thrombin to the platelet suspensions, they were preincubated with various concentrations of metformin (2–10 mM) or PBS (an isovolumetric solvent control) for 3 min. The reaction was allowed to proceed for 6 min, and the extent of aggregation was quantified in light transmission units. To measure the ATP release, a luciferin-luciferase mixture (20 µL) was added 1 min before the addition of agonists, and the amount of ATP released in the experimental group was compared with that of the control using an F-7000 spectrometer (Hitachi, Tokyo, Japan) following the manufacturer's instructions.

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

The supernatant collected from the centrifuged citrated whole blood was subjected to an incubation step with Fura 2-AM (5 µM) for 1 h. Human platelets were prepared andadjusted to have 1 mM Ca^{2+} concentration. The relative intracellular Ca^{2+} ion concentration ($[Ca^{2+}]i$) was measured using excitation wavelengths of 340 and 380 nmand an emission wavelength of 500 nm [15].

Additionally, washed platelets were preincubated with either PBS or metformin (5 and10 mM), along with FITC-conjugated anti-P-selectin monoclonal antibody (2 µg/mL),for 3 min. Subsequently, these platelets were stimulated with thrombin (0.02 U/mL). The fluorescence-labeled platelets were then identified in the suspensions using a flow cytometer (FACScan system; Becton Dickinson, San Jose, CA, USA). Data were collected from approximately 50,000 platelets in each experimental group, and the

platelets were recognized based on their forward and orthogonal light-scattering characteristic profiles.

Immunoblotting

The platelet suspensions were initially treated with metformin at concentrations of 5 and 10 mM, as well as PBS, and then thrombin (0.02 U/mL) was added to induce platelet activation. After the reaction was completed, the platelets were directly resuspended in 200 µL of a lysis buffer containing HEPES (50 mM, pH 7.4), NaCl (50mM), Triton X-100 (1%), EDTA (5 mM), and various supplements including leupeptin(2 µg/mL), aprotinin (10 µg/mL), sodium pyrophosphate (5 mM), NaF (10 mM), PMSF (1 mM), and sodium orthovanadate (1 mM). The samples were incubated in the lysis buffer for 2 h. Lysates with 60 µg of protein were separated using 12% sodium dodecylsulfate 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 (diluted1:1000 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 protiens was calculated after normalized to their respective total proteins.

Statistical Analysis

The results are presented as mean ± standard error of the mean, and the value of *n* represents the number of experiments conducted in this study. Differences among the experimental groups were analyzed using one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls posthoc test. A p-value of less than 0.05 was considered statistically significant. Statistical analysis was performed using SAS Version 9.2 (SAS, Cary, NC, USA).

Results

Metformin's Effect on Inhibiting Human Platelet Aggregation and Granule Secretion

Figure 1 demonstrates the concentration-dependent suppression of thrombin (0.02 U/mL)-induced platelet aggregation in washed human platelets by metformin (2–10 mM). P-selectin serves as a crucial biomarker for platelet activation. In its normal state, P-selectin is expressed on the inner face of α-granules. However, when platelets are activated, they expose the inner face of the granules to the outer parts of the cells [16].

Metformin significantly reduced thrombin-stimulated FITC-P-selectin expression in the flow cytometry study (Figure 2A). The results are depicted in the right panel of Figure 2A (a, resting control [Tyrode's solution], 161.3 ± 24.8; b, collagen-activated platelets, 1833.7 ± 93.6; c, metformin 5 mM, 1315.7 ± 55.2; d, 10 mM, 636.3 ± 160.5;n = 4). Furthermore, several lines of evidence support the direct association between platelet activation and the release of granular contents, including Ca^{2+} and ATP. This release triggers a significant increase in platelet aggregation, leading to strong platelet activation. As presented in Figure 2B and C, metformin significantly suppressed both the $[Ca^{2+}]i$ mobilization and ATP-release reaction stimulated by thrombin. The relative data are expressed as inhibition percentages on the right hand side of each figure (B, $[Ca^{2+}]i$ mobilization: metformin 5 mM, 78.0 ± 7.0%; 10 mM, 47.5 ± 10.1%, n = 4; C, ATP-release reaction: metformin 5 mM, 60.1 ± 13.2%; 10 mM, 26.3 ± 6.4%, n = 4).

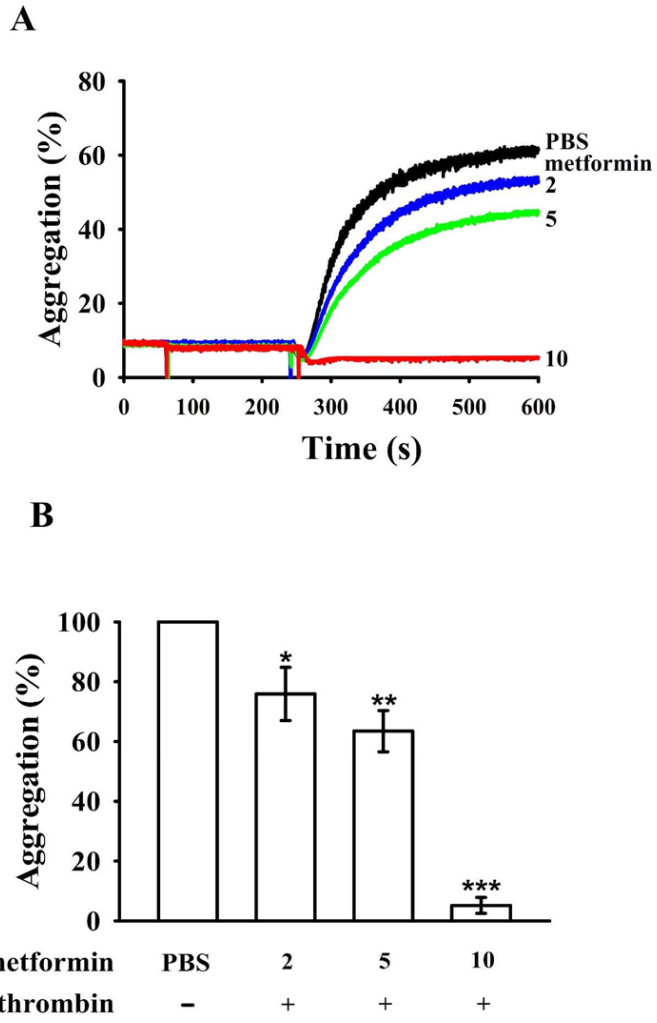

Figure 1: Inhibition of thrombin-induced platelet aggregation by metformin. (A) Washed human platelets were preincubated with either a solvent control (PBS) or various concentrations of metformin (2, 5, and 10 mM) before being stimulated with thrombin (0.02 U/mL) to induce platelet aggregation. The bar diagrams in (B) illustrate the corresponding statistical analyses. The data are expressed as the mean ± standard errorof the mean ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs PBS + thrombin group.

Regulatory Activity of PKC and Akt by Metformin

PKC and Akt (protein kinase B) are two important signaling molecules involved in the activation of platelets, and their relationship with thrombin plays a crucial role in platelet function [17]. Thrombin, a key enzyme in the coagulation cascade, activates platelets through the protease-activated receptors (PARs) on the platelet surface. Uponbinding of thrombin to PARs, it leads

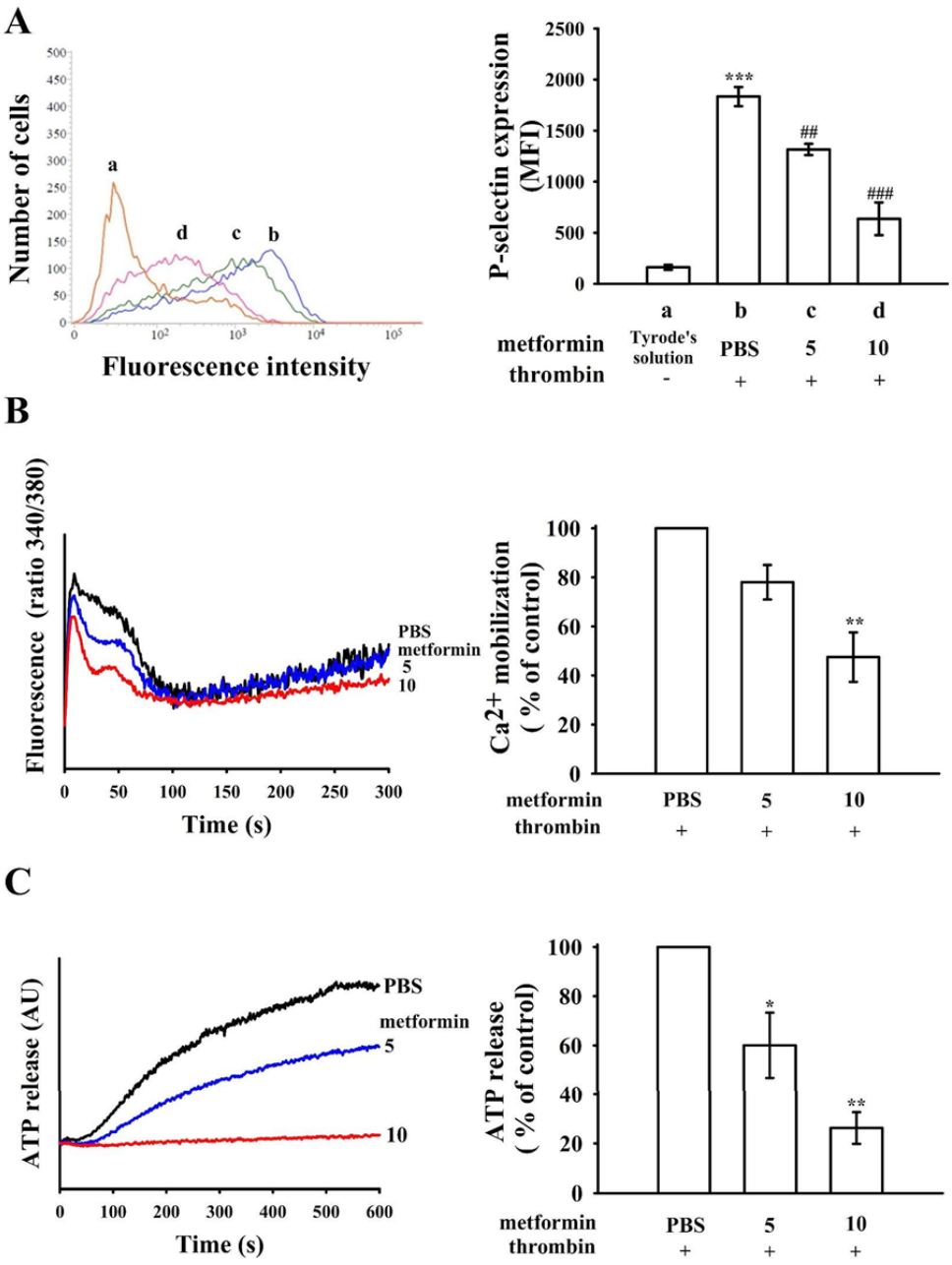

Figure 2: Metformin's inhibitory effects on surface P-selectin expression, relative $[Ca^{2+}]i$ mobilization, and ATP-release in human platelets. Washed platelets were preincubatedwith either PBS or metformin (5 and 10mM). Following preincubation, platelets were treated with thrombin (0.02 U/mL) to induce platelet activation. (A) Surface P-selectinexpression (MFI; mean fluorescence intensity) is depicted in fluorescence images as follows: a) Tyrode's solution, b) collagen-activated, c) metformin 5 mM, and d) metformin 10 mM. (B) Relative $[Ca^{2+}]i$ mobilization and (C) ATP release (AU; arbitrary unit). The corresponding statistical analyses are presented in the bar diagrams.Results are expressed as mean ± standard error of the mean (n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs PBS + Tyrode's solution (A) or thrombin group (B and C); $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$vs PBS + thrombin group (A).

to the activation of PKC and subsequent activation of Akt. Akt activation is mediated by PKC-dependent phosphorylation events [18]. Activated Akt then regulates various downstream signaling pathways involved inplatelet activation, such as granule secretion, integrin activation, and cytoskeletal rearrangement. Metformin decreased significantly PKC (p-p47) and Akt activation in thrombin-activated platelets (Figure 3A and B). These results uggest that metformin blocks PKC/Akt signaling pathway.

Influence of Metformin in MAPKs Activation

The MAPK pathway serves as a crucial mediator in transmitting thrombin signals and orchestrating platelet responses during hemostasis and thrombus formation. Metformin (5 and 10 mM) reduced p38 MAPK, while ERK1/2 and JNK1/2 phosphorylation were slightly inhibited but not significantly. These findings strongly suggest that the p38 MAPK molecule plays a crucial role in mediating the antiplatelet effects of metformin(Figure 4). Consequently, the inhibition of p38 MAPK activation appears to

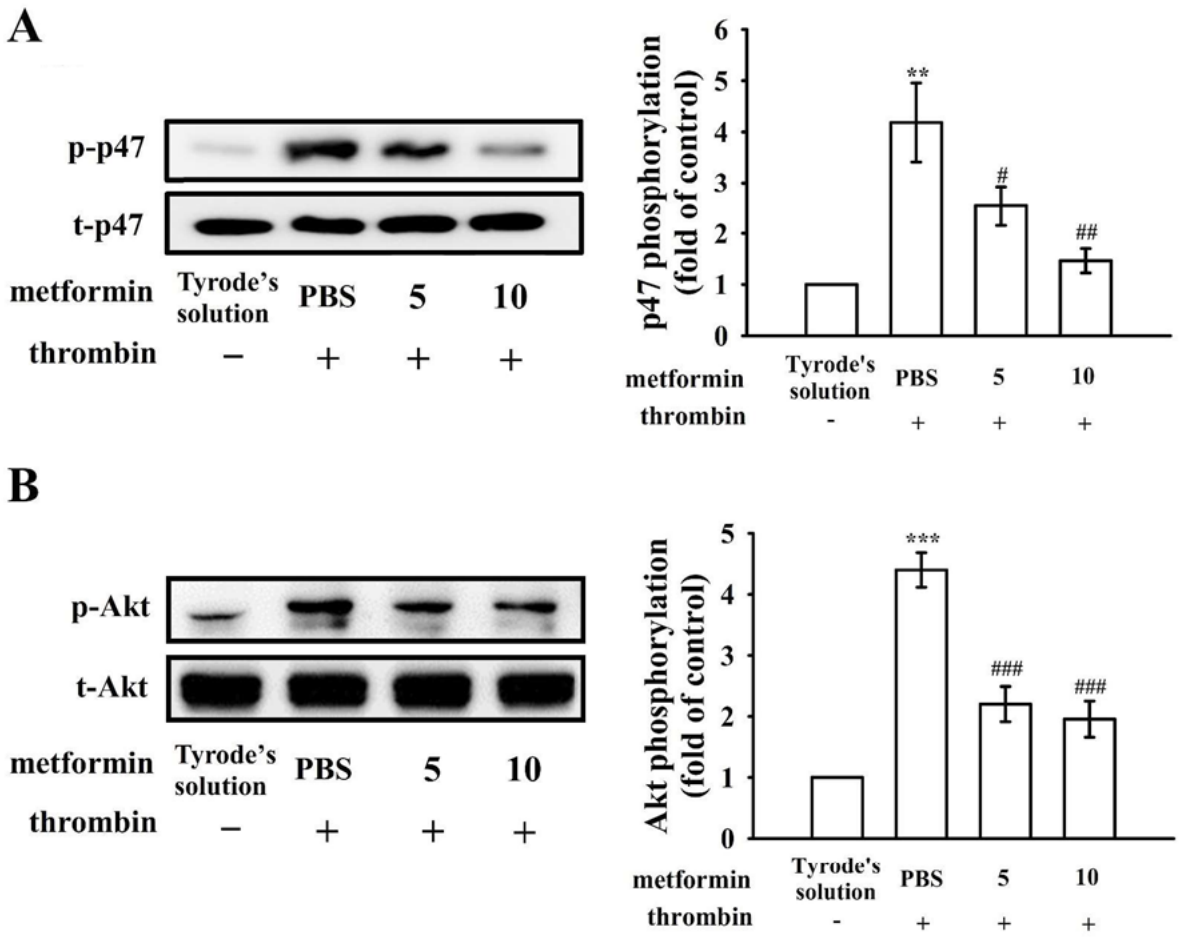

Figure 3: Regulatory effect of metformin in protein kinase C (PKC) and Akt. Washed platelets were preincubated with either PBS or metformin (5 and 10 mM), followed by treatment with thrombin (0.02 U/mL) for immunoblotting analysis of (A) PKC (p-47) and (B) Akt phosphorylation. Results are expressed as mean ± standard error of the mean ($n = 4$). $^{**}p < 0.01$ and $^{***}p < 0.001$ vs Tyrode's solution; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs PBS + thrombin group.

be a critical factor contributing to the antiplatelet effects observed with metformin treatment.

Discussion

In our studies, we have found that metformin could inhibit collagen [13] and thrombin- induced platelet activation to prevent thrombotic-related diseases. Studies have shown that metformin can influence various cellular processes, including mitochondrial function, inflammation, and oxidative stress, all of which are closely connected to nutrient sensing and metabolism [19,20]. Additionally, metformin has been associated with the modulation of gut microbiota, a critical factor in nutrient absorption and metabolism [21]. Furthermore, preclinical and clinical studies have demonstrated that metformin supplementation may mimic certain physiological responses triggered by caloric restriction, leading to improvements in lifespan and age-related diseases [22]. These findings provide intriguing insights into the potential of metformin as a nutrient agent, highlighting its multifaceted effects on cellular and metabolic processes beyond its antidiabetic properties.

Platelet activation is triggered by different agonists, including collagen, thrombin, AA, U46619, which show their effects through interactions with specific receptors on platelet membranes. In this study, metformin has effective on thrombin-induce platelet aggregation. The findings of this study shed light on the critical role of thrombin in platelet activation and hemostasis. Thrombin, a serine protease, plays a crucial role in platelet activation and hemostasis. Upon vascular injury, thrombin is generated through the coagulation cascade, leading to the conversion of fibrinogen to fibrin and the formation of a stable blood clot. Thrombin interacts with platelets through protease- activated receptors (PARs), specifically PAR-1 and PAR-4, expressed on the platelet surface [17]. The binding of thrombin to PARs induces conformational changes, leading to intracellular signaling cascades. Thrombin activates several downstream pathways in platelets, including the phospholipase C (PLC), PKC, MAPKs and RhoA/Rho kinase pathways [23]. These pathways contribute to platelet shape change, granule secretion, and integrin activation. Platelet shape change involves reorganization of the actin cytoskeleton, leading to platelet spreading and the formation of filopodia and lamellipodia. The intricate mechanism of thrombin-platelet interaction emphasizes the interplay of signaling pathways and molecular events that regulate platelet activation and contribute to hemostasis.

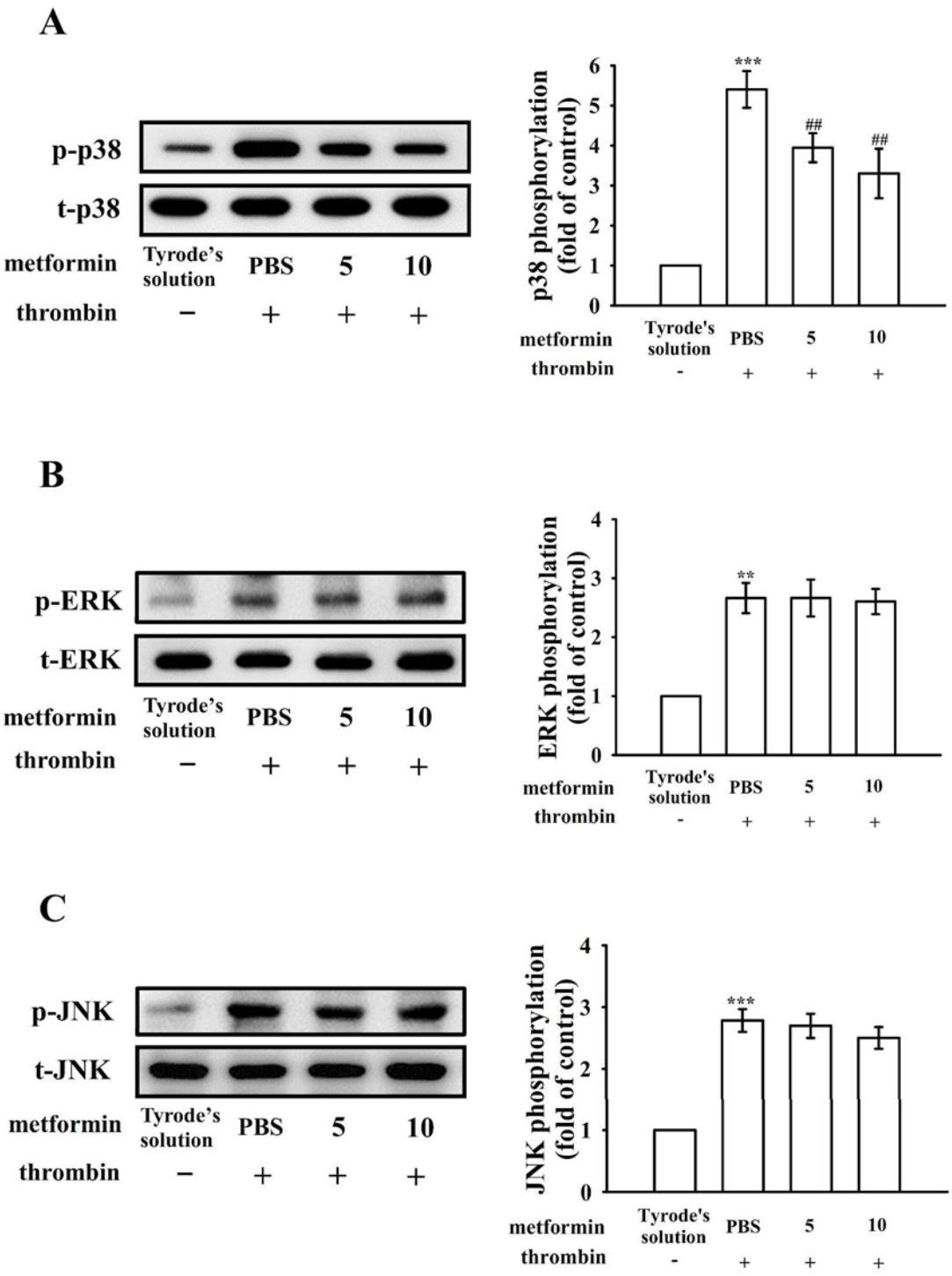

Figure 4: Effect of metformin on mitogen-activated protein kinase (MAPK) phosphorylation in platelets. Washed platelets were preincubated with either PBS or metformin at concentrations of 5 and 10 mM, followed by treatment with thrombin (0.02 U/mL). Immunoblotting was performed to assess the phosphorylation levels of (A) p38 MAPK, (B) ERK1/2, and (C) JNK1/2. Results are expressed as the mean ± standard error of the mean (n = 4). $^{**}p < 0.01$ and $^{***}p < 0.001$ vs Tyrode's solution; $^{\#\#}p < 0.01$ vs PBS + thrombin group.

Thrombin binding to its receptor, PARs, leads to the activation of phospholipase C (PLC), resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers calcium release from intracellular stores, leading to a rise in intracellular calcium levels [23]. DAG, in conjunction with calcium, activates PKC isoforms. Activated PKC phosphorylates a variety of substrates, including key proteins involved in platelet activation. One of the critical targets of PKC is the integrin $\alpha IIb\beta 3$, which undergoes conformational changes and shifts to a high-affinity state for fibrinogen binding, thereby promoting platelet aggregation [24]. This study found that metformin reduced thrombin-induced

PKC activation, suggesting that PKC plays a crucial role in thrombin-induced plateletactivation.

Three isoforms of Akt (Akt1, Akt2, and Akt3) are expressed in platelets [25], and knockout of any one of them reduces low-dose thrombin- and U46619-induced platelet secretion and the secretion-dependent second-wave aggregation [23]. Kroner et al. reported that thrombin-dependent phosphorylation of Akt^{Ser473} in platelets was not completely inhibited by the PI3K inhibitor LY294002 but was substantially inhibited by inhibitors of PKC isoforms α or β [17]. We noted that PKC and Akt phosphorylation were abolished by metformin. This cross-talk between PKC and Akt signaling pathways exemplifies the intricate coordination of intracellular events involved in thrombin- induced platelet activation and highlights the significance of PKC-mediated Akt activation in regulating platelet function.

Activation of the MAPK pathway involves three major kinases: ERK, JNK, and p38 MAPK. Thrombin induces the phosphorylation and activation of these MAPKs, leading to the regulation of various platelet responses [26]. The physiopathological roles of JNKs and ERKs in platelets remain unclear, but might entail the suppression of αIIbβ3 integrin activation or the negative regulation of platelet activation [27]. Nevertheless, p38 MAPK provides a crucial signal for platelet activation [28]. Among the numerous downstream targets of p38 MAPK, the most physiologically relevant in platelets is cytosolic phospholipase A2, which catalyzes arachidonic acid release to produce TxA2 [28]. Moreover, other study indicated that activation of p38 MAPK, which in turn initiates NF-κB activation, and ultimately induces platelet activation by thrombin [14]. This study demonstrated that p38 MAPK, but not ERKs or JNKs, activation is inhibited by metformin in thrombin-induced human platelets. p38 MAPK pathway is essential for the generation of pro-inflammatory mediators and cytokines in platelets, linking thrombin signaling to various inflammatory processes.

Conclusion

Metformin efficiently diminished platelet activation by interfering with the PKC/Akt and p38 MAPK signaling pathways stimulated in humans. Because of the widespread use of metformin and its well-established safety and no toxicity profile, it could serve as a promising nutritional agent for CVDs or other chronic diseases when used in the long term at a low dose.

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References

1. Sheu JR, Yen MH, Hung WC, et al. Triflavin Inhibits Platelet-Induced Vasoconstriction in de-Endothelialized Aorta. Arterioscler Thromb Vasc Biol. 1997; 17: 3461-3468.
2. Stegner D, Nieswandt B. Platelet Receptor Signaling in Thrombus Formation. J Mol Med. 2011; 89: 109-121.
3. Shin JI, Sang Y, Chang AR, et al. 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.
4. 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.
5. Bai B, Chen H. Metformin: A novel weapon against inflammation. Front Pharmacol. 2021; 12: 622262.
6. Salvatore T, Pafundi PC, Galiero R, et al. Can metformin exert as an active drug on endothelial dysfunction in diabetic subjects? Biomedicines. 2020; 9: 3.
7. Anfossi G, Russo I, Bonomo K, et al. 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.
8. Xin G, Wei Z, Ji C, et al. Metformin uniquely prevents thrombosis by inhibiting platelet activation and mtDNA release. Sci Rep. 2016; 6: 36222.
9. Gin H, Freyburger G, Boisseau M. et al. Study of the effect of metformin on platelet aggregation in insulin-dependent diabetics. Diabetes Res Clin Pract. 1989; 6: 61-67.
10. De Caterina R, Marchetti P, Bernini W, et al. The direct effects of metformin on platelet function *in vitro*. Eur J Clin Pharmacol. 1989; 37: 211-213.
11. Gargiulo P, Caccese D, Pignatelli P, et al. Metformin decreases platelet superoxide anion production in diabetic patients. Diabetes Metab Res Rev. 2002; 18: 156-159.
12. Formoso G, De Filippis EA, Michetti N, et al. 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.
13. Chang Y, Huang WC, Hsu CY, et al. Metformin serves as a novel drug treatment for arterial thrombosis: inhibitory mechanisms on collagen-induced human platelet activation. Appl Sci. 2022l; 12: 7426.
14. Chen WF, Lee JJ, Chang CC, et al. Platelet protease-activated receptor (PAR)4, but not PAR1, associated with neutral sphingomyelinase responsible for thrombinstimulated ceramide-NF-κB signaling in human platelets. Haematologica. 2013; 98: 793-801.
15. Sheu JR, Lee CR, Lin CH, et al. Mechanisms involved in the antiplatelet activity of staphylococcus aureus lipoteichoic acid in human platelets. Thromb Haemost. 2000; 83: 777-784.
16. Merten M, Thiagarajan P. P-selectin expression on platelets determines size and stability of platelet aggregates. Circulation. 2000; 102: 1931-1936.
17. Offermanns S. Activation of platelet function through G protein-coupled receptors. Circ Res. 2006; 99: 1293-1304.

18. Woulfe DS. Akt signaling in platelets and thrombosis. Expert Rev Hematol. 2010; 3: 81-91.

19. Lv Z, Guo Y. Metformin and its benefits for various diseases. Front Endocrinol (Lausanne). 2020; 11: 191.

20. Chen S, Gan D, Lin S, et al. Metformin in aging and aging-related diseases: clinical applications and relevant mechanisms. Theranostics. 2022; 12: 2722-2740.

21. Lee H, Lee Y, Kim J, et al. Modulation of the gut microbiota by metformin improves metabolic profiles in aged obese mice. Gut Microbes. 2018; 9: 155-165.

22. Martin-Montalvo A, Mercken EM, Mitchell SJ, et al. Metformin improves healthspan and lifespan in mice. Nat Commun. 2013; 4: 2192.

23. Estevez B, Du X. New concepts and mechanisms of platelet activation signaling. Physiology (Bethesda). 2017; 32: 162-177.

24. Moore SF, van den Bosch MT, Hunter RW, et al. Dual regulation of glycogen synthase kinase 3 (GSK3)α/β by protein kinase C (PKC)α and Akt promotes thrombin-mediated integrin αIIbβ3 activation and granule secretion in platelets. J Biol Chem. 2013; 288: 3918-3928.

25. Laurent PA, Severin S, Gratacap MP, et al. 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.

26. Bugaud F, Nadal-Wollbold F, Lévy-Toledano S, et al. Regulation of c-jun-NH2 terminal kinase and extracellular-signal regulated kinase in human platelets. Blood. 1999; 94: 3800-3805.

27. Hughes PE, Renshaw MW, Pfaff M, et al. Suppression of integrin activation: a novel function of a Ras/Raf-initiated MAP kinase pathway. Cell. 1997; 88: 521-530.

28. Coulon L, Calzada C, Moulin P, et al. Activation of p38 mitogen-activated protein kinase/cytosolic phospholipase A2 cascade in hydroperoxide-stressed platelets. Free Radic Biol Med. 2003; 35: 616-625.