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RESEARCH ARTICLE

Hexokinase controls platelet activation and hemostasis

Lih T. Cheah, Jawad S. Khalil, Beth A. Webb, Daisie M. Yates, Reem Alotaibi*, Mary McKay, Mohammad Ali, Thomas Palmer-Dench, Emily Horner, Mugthalana Allet, Fraser Macrae, Cedric Duval, Amanda J. Unsworth, Mark T. Kearney, and Khalid M. Naseem

Leeds Institute of Cardiovascular and Metabolic Medicine, University of Leeds, Leeds, UK

ABSTRACT

Platelet activation is energy-dependent and associated with a hyperglycolytic phenotype, yet the role of key glycolytic enzymes remains unclear. We investigated the role of platelet hexokinases (HK1 and HK2) in regulating thrombin- and convulxin-stimulated bioenergetics, functions, and hemostasis using pharmacological inhibitors 2-deoxyglucose (2DG; pan-HK) and 3-bromopyruvate (3BP; HK2-preferential). Platelet activation increased HK activity, with convulxin producing a stronger response than thrombin. Using 2DG and 3BP, we found that activation-induced glucose uptake, glycolytic ATP production, and glycolysis were dependent on both HK isoforms. Platelet aggregation was reduced but not abolished by HK inhibition, indicating that platelet function is underpinned by metabolic flexibility. Integrin activation and pro-coagulant platelet formation were suppressed by both inhibitors. In contrast, α-granule secretion markers (CD62P, CD40L), platelet spreading, and convulxin-induced reactive oxygen species (ROS) generation were more sensitive to 3BP, suggesting a prominent role for HK2 in secretion, outside-in signaling, and ROS regulation. *In vitro* thrombus formation over collagen was significantly reduced by both inhibitors. In addition, HKs play a pivotal role in the regulation of *in vivo* hemostasis with increased bleeding time in mice treated with HK inhibitor. These findings identify HK as a central regulator of platelet metabolism and function, with distinct contributions of HK isoforms to specific platelet responses.

PLAIN LANGUAGE SUMMARY

What is in the context?

- Platelets are blood cells that help stop bleeding by forming clots.
- Platelet activation requires energy for processes including shape change, granule secretion, and aggregation.
- Platelets mainly generate this energy through glycolysis, a pathway that uses glucose.
- Platelet activation is known to increase glycolysis, but the role of specific glycolytic enzymes in regulating platelet function is not fully understood.

What is new?

- This study identifies hexokinase, a key enzyme in glucose metabolism, as an important regulator of platelet activation.
- Platelet stimulation with thrombin and convulxin increases glucose uptake and glycolysis through hexokinase-dependent mechanisms.
- Pharmacological inhibition of hexokinase reduces platelet aggregation, ROS generation, granule secretion, procoagulant platelet formation, spreading, and thrombus formation *in vitro*.

What is the impact?

- Hexokinase plays a critical role in regulating platelet metabolism and function.
- Targeting hexokinase may represent a potential metabolic strategy to reduce excessive platelet activation.
- This approach may help develop new therapies for thrombotic diseases.

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CONTACT Lih T. Cheah l.t.cheah@leeds.ac.uk Leed sInstitute of Cardiovascular & Metabolic Medicine, Universityof Leeds, The LIGHT Laboratories, Clarendon Way, Leeds LS2 9NL, UK

*Current address: Department of Biochemistry, College of Science, King Saud University, Riyadh, Saudi Arabia.

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Introduction

Platelets play vital roles in the pathophysiology of acute myocardial infarction and ischemic stroke following acute coronary syndrome (ACS), with anti-platelet therapy pivotal in preventing thrombotic complications in patients with ACS.[1–3] Current anti-platelet drugs, including P2Y12 inhibitors and aspirin, have been shown to give improved clinical outcomes in ACS patients. Nevertheless, some patients continue to experience adverse ischemic events as platelets remain activated via alternative pathways[4] and are at an increased risk of bleeding complications.[5–7] These limitations have prompted interest in understanding the cellular and metabolic mechanisms that regulate platelet activation, with the goal of identifying complementary strategies to modulate platelet function without exacerbating bleeding risks.

Platelets are highly metabolically active cells.[8,9] They circulate in the bloodstream in a quiescent state but must undergo rapid activation upon vascular injury. Key platelet functions, including morphological changes, granule secretions, and aggregation, require a rapid increase in the ATP availability.[10–12] Studies from our group and others have demonstrated that resting platelets exhibit a metabolic plasticity, enabling flexible fuel selection. In contrast, activated platelets adopt a highly glycolytic phenotype,[8,13–15] regardless of nutrient availability. The switch to glycolysis is a similar phenomenon to the Warburg effect, a hallmark of cancer,[16] and is consistent with the metabolic reprogramming observed in other myeloid cells.[17] In dendritic cells and macrophages, the increased reliance on glucose leads to distinct cellular functions in innate immunity.[17] For example, classically activated macrophages and dendritic cells utilize glycolysis to promote inflammation by releasing the inflammatory cytokine IL-1β,[18] as well as for its maturation and function in response to LPS.[19] The discovery of the link between the rewiring of glucose metabolism in cancer and immune cells has led to the exploration of the targeting key metabolic enzymes as a therapeutic strategy. Indeed, there are several studies that have highlighted the key roles of metabolic enzymes such as pyruvate kinase M2 (PKM2)[20] and pyruvate dehydrogenase kinase (PDK)[21] in driving platelet aggregation and thrombosis. However, the importance of the rate-limiting steps in glycolysis remains unclear.

The commitment of glucose to glycolysis requires its conversion to glucose-6-phosphate by hexokinases (HKs). Proteomic and transcriptomic studies indicate expression of HK1 and HK2 in human and murine platelets.[22,23] The presence of multiple HK isoforms raises the question of whether they mediate distinct functions, as different HK isoforms differ in catalytic properties, regulatory features, and subcellular localization.[24] The present study was undertaken to determine the roles of HK, in the regulation of platelet energy metabolism, activation, aggregation, and thrombus formation, with the aim of defining how these rate-limiting enzymes contribute to platelet function.

Materials and methods

Materials

PP2, PP3, HK activity assay kit, and 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose (2-NDBG) glucose uptake kit were from Abcam (Cambridge, UK). 2DG, thrombin, CellLytic™ MT cell lysis reagent, protease cocktail inhibitor, phosphatase cocktail inhibitor, and protein G Sepharose beads were from Sigma (Dorset, UK). 3BP was from Selleckchem (Houston, USA). Wortmannin, prostacyclin I_2 (PGI_2), convulxin, and Bay61-3606 were from Cayman Chemical (Michigan, USA). Collagen was from Takeda (London, UK). Bovine serum albumin (BSA) was from Roche (West Sussex, UK). 3,3′-dihexyloxacarbocyanine iodide (DIOC6) was from Calbiochem (Dorset, UK). Dulbecco's modified Eagle medium (DMEM), Seahorse XF real-time ATP rate assay kit, and glycolysis stress test kit were from Agilent Technologies (Cheadle, UK). Antibodies to APC/Cyanine7-conjugated CD42b, AF700-conjugated CD62p, FITC-conjugated PAC1, PE/Dazzle™ 594-conjugated CD40L, and Brilliant Violet 650™-conjugated CD63 were from Biolegend (Cambridge, UK). Rabbit Anti-HK1, HK2, and GAPDH antibodies were from Cell Signalling Technology (Leiden, The Netherlands). APC-conjugated Annexin V and FITC-labelled phalloidin were from ThermoFisher Scientific (Milton, UK). ROS-ID Total ROS detection kit was from Enzo (New York, USA).

Bioenergetic profiles in platelets

The Agilent Seahorse XFe96 Analyzer (Agilent Technologies) was used to measure platelet oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) as previously described.[13,25] 2-deoxyglucose (2DG, 50 mM), 3-bromopyruvate (3BP, 30 μM), or DMEM buffer–treated washed platelets (WP, 2×10^8 platelets/ml, 50 μl) were seeded in a cell-culture miniplate. The ATP rate assays were performed as previously described.[25] The glycolysis stress test was initiated by sequential injections of glucose (5 mM), convulxin/thrombin/DMEM, oligomycin (1 μM), and 2DG (50 mM), according to the manufacturers' instructions (Agilent Technologies).

Measurement of glucose uptake

The fluorescently labeled glucose analogue, 2-NBDG, was used to measure glucose uptake in platelets. In brief, WP (2.5×10^7 platelets/ml) were activated with convulxin or thrombin (of various concentrations) for 10 minutes, followed by staining with 375 μM 2-NBDG before being analyzed using a Beckman Coulter CytoFLEX RUO flow cytometer (Amersham, UK).

Hexokinase activity

WP (5×10^8/ml) were pre-treated with 2DG (50 mM) or 3BP (30 μM) or modified Tyrode's buffer for 15 minutes followed by agonist treatment for 30 minutes. Then, an equal volume of CelLytic™ MT cell lysis reagent containing protease and phosphatase inhibitors was added to the platelet suspension and rested on ice for 30 minutes, before centrifugation at 14 000 *g* for 2 minutes at 4°C. Platelet cytosolic fractions were aliquoted for HK activity assays, which were carried out according to the manufacturers' instructions. In some cases, WP were pre-treated with PP2 (20 μM), PP3 (20 μM), Bay61-3606 (10 μM), or Wortmannin (200 nM) for 20 minutes before activation.

Platelet isolation, light transmission platelet aggregation, ROS generation, flow cytometric expression of platelet activation markers, immunoprecipitation and immunoblotting, adhesion and spreading, *in vitro* thrombosis, experimental animals, tail bleeding time, and hemoglobin assay.

Detailed methods are presented in the Supplemental Methods.

Statistical analysis

Results are expressed as mean ± SD, and statistical analyses were undertaken using Prism 9.5 (GraphPad). Comparisons between groups were performed by unpaired t-test with Welch's correction, one-way ANOVA, or two-way ANOVA. *p* values of less than 0.05 were considered statistically significant.

Results

The presence of hexokinase 1 and 2 in human platelets

Previous studies have shown the presence of HK1 and HK2 in murine platelets, and their expression in human platelets has been predicted by transcriptomic studies.[22,23] Using immunoprecipitation with isoform-specific antibodies, we detected the expression of HK1 and HK2 in human platelets (Figure 1A), corroborating previous proteomic and biochemical studies.[22,23] We next examined HK activity in platelets at rest and in response to activation by the GPVI agonist convulxin or thrombin. Resting HK activity in platelet lysates was 0.76 ± 0.40 mU/ml and was increased significantly in response to convulxin (11.51 ± 2.68 mU/ml; Figure 1B) and to a lesser degree to thrombin (2.25 ± 0.55 mU/ml; Figure 1C), even though these agonists both induce maximal aggregation. The larger HK activity increase with convulxin likely reflects pathway-specific differences rather than a difference in activation magnitude. In the absence of genetic models, we decided to examine the potential importance of HKs in platelet function using a pharmacological approach, employing an established pan-HK inhibitor, 2DG[26,27] and 3BP which has a preference for HK2.[28] Preincubation of platelets with 2DG (50 mM) or 3BP (30 μM) led to a significant inhibition, but not ablation, of both convulxin- and thrombin-inhibited HK activity (Figures 1B and 1C).

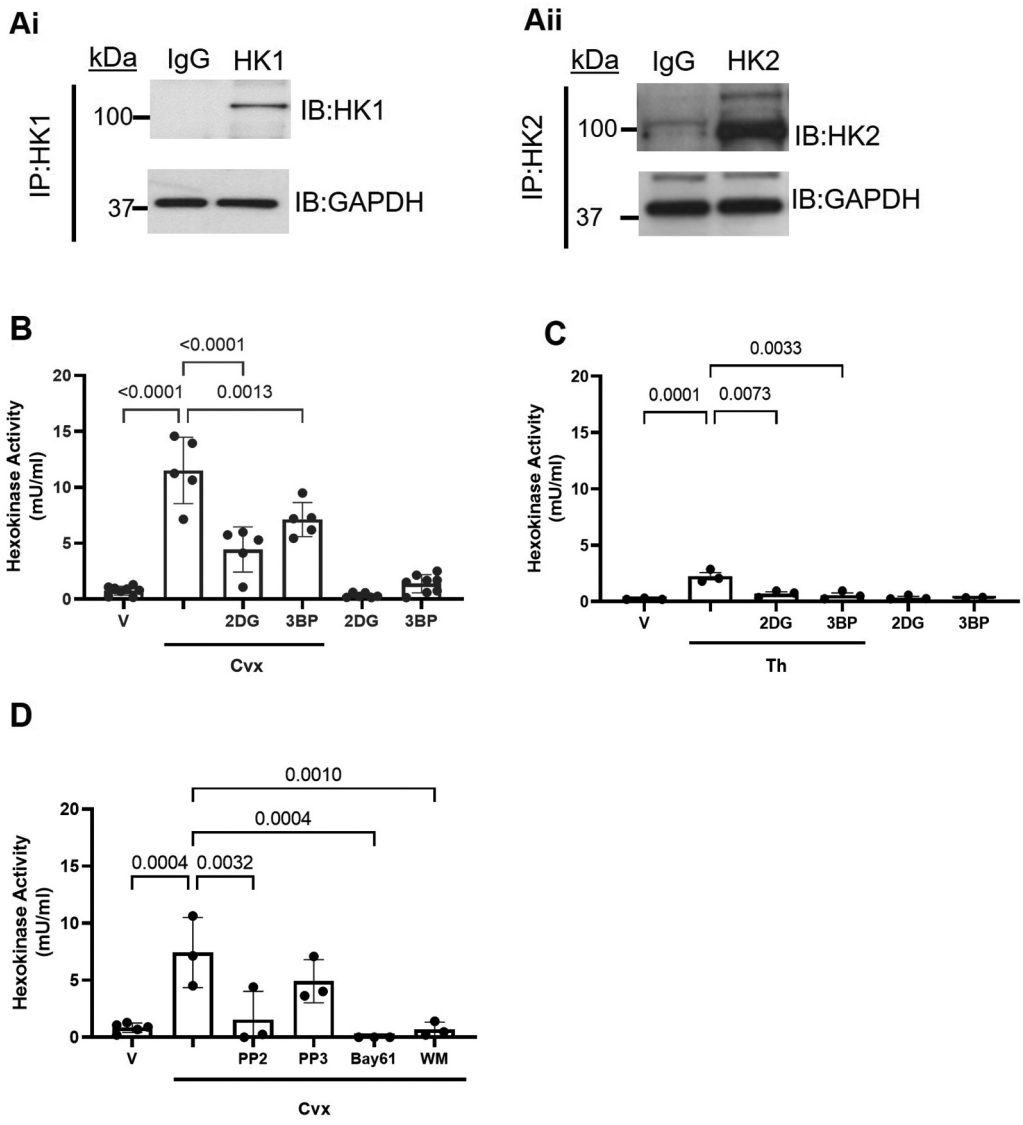

Figure 1. Expression and regulation of hexokinase in human platelets. (A) Immunoprecipitation of HK1 (Ai) or HK2 (Aii) from WP, followed by immunoblotting for HK1/HK2 and GAPDH (loading control). (B-C) Hexokinase activity in WP pre-treated with vehicle, 2DG (50 mM) or 3BP (30 μM) and stimulated with vehicle (V) or convulxin (Cvx, 50 ng/ml) or thrombin (Th, 0.05 U/ml). (D) Hexokinase activity in WP pre-treated with Src family kinase inhibitor (PP2), inactive analogue (PP3), Syk inhibitor (Bay61-3606), or PI3K inhibitor (WM), followed by convulxin stimulation. Data represent mean ± SD ($n = 5$ for B-C; $n = 3$ for D). Statistical analysis was performed using one-way ANOVA.

Given that GPVI stimulation caused a large increase in HK activity, we confirmed that activation was linked to GPVI signaling via tyrosine kinases. When Src family kinases were inhibited by PP2, the convulxin-induced HK activity was significantly reduced to basal level while the inactive analogue PP3 had significant effect on HK activity (Figure 1D). Inhibition of Syk with BAY61-3606 similarly reduced HK activity to the basal levels. In addition, HK has been shown to be regulated by phosphoinositide 3-kinase (PI3K), which also plays a key role in GPVI-mediated platelet activation.[29] Inhibition of PI3K using Wortmannin also significantly reduced GPVI-mediated HK activity (Figure 1D). These data suggest that nodes within the GPVI signaling pathway may drive HK to promote glycolysis during platelet activation.

Effect of platelet agonists on glucose uptake and metabolism

Since HK catalyzes the first step of glucose metabolism in glycolysis, we investigated the effects of platelet agonists on glucose metabolism. Stimulation of platelets with convulxin (Figure 2Ai) or

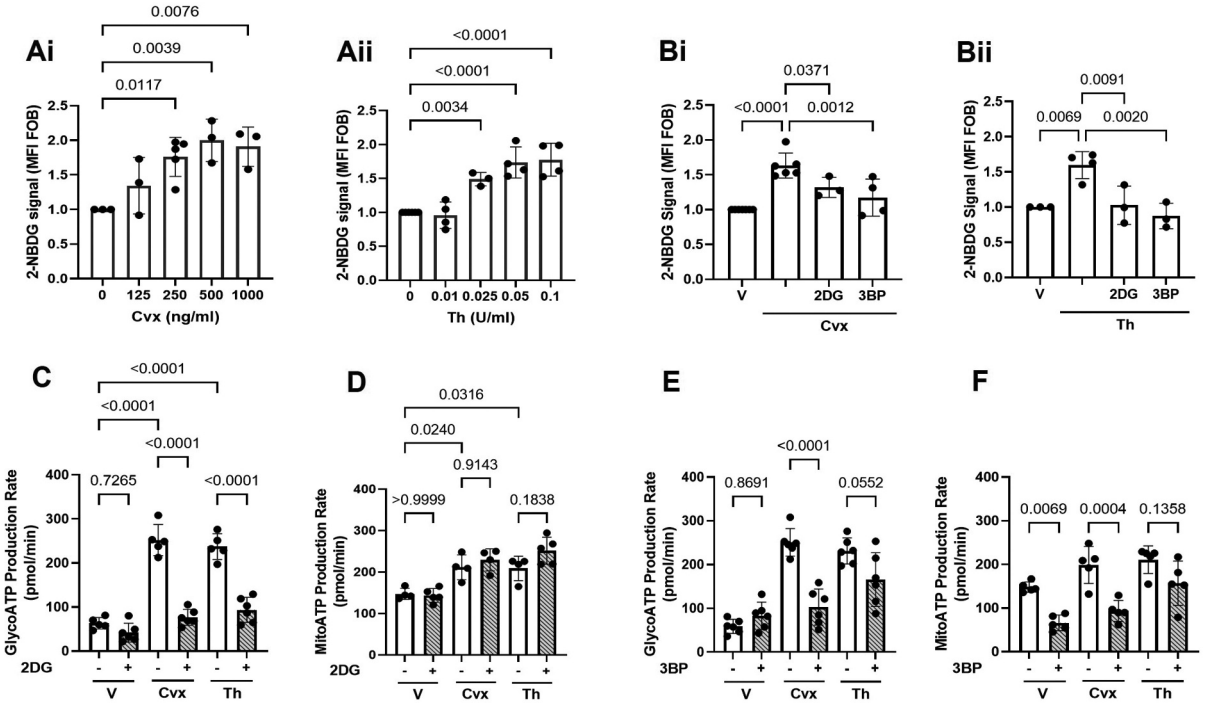

Figure 2. Effects of platelet agonists on glucose uptake and ATP production. (A) Glucose uptake (2-NBDG) in WP stimulated with increasing concentrations of convulxin (Cvx, Ai) or thrombin (Th, Aii). (B) Glucose uptake (2-NBDG) in WP pre-treated with vehicle, 2DG (50 mM) or 3BP (30 μM) and stimulated with vehicle (V), convulxin (250 ng/ml) or thrombin (0.05 U/ml). Data are expressed as fold change over basal fluorescence (n = 3–6). (C-F) GlycoATP (C, E) and mitoATP (D, F) production rates in WP pre-treated with vehicle or 2DG (C-D) or 3BP (E-F), followed by stimulation with vehicle (V), convulxin (250 ng/ml), or thrombin (0.05 U/ml) (n = 5–6). Data represent mean ± SD. Statistical analysis was performed using one-way ANOVA.

thrombin (Figure 2Aii) induced dose-dependent accumulation of 2-NBDG indicating increased glucose uptake during activation. Preincubation of platelets with 2DG or 3BP almost completely abolished glucose uptake (Figure 2B) consistent with a role for HK in maintaining the concentration gradient that drives glucose uptake. To confirm this commitment of glucose to metabolism, ATP production rates from the two major energy-producing pathways, glycolysis and oxidative phosphorylation (OXPHOS), were measured. Convulxin (50 ng/ml) and thrombin (0.03 U/ml) increased glycoATP production rate from 63.17 ± 13.09 pmol/min to 251.98 ± 35.09 pmol/min ($p < .0001$) and 237.09 ± 29.28 pmol/min ($p < .0001$), respectively. In the presence of 2DG, glycolysis remained at basal levels (Figure 2C). While mitochondrial respiration (OCR) remains unchanged following agonist stimulation (Supplementary Figure 1), mitoATP production rate showed a modest, but significant increase. This increase was unaffected by inhibiting glycolysis with 2DG (Figure 2D), suggesting that mitochondrial ATP generation is maintained independently of glycolytic flux. In contrast, treatment with 3BP, which only inhibits HK2, significantly reduced both glycoATP and mitoATP production rates in convulxin-stimulated platelets (Figures 2E and 2F). However, 3BP did not affect thrombin-stimulated metabolic pathways. These findings may hint that the contribution to HK2 to platelet bioenergetics may be stimulus dependent.

Given the clear importance of glycoATP to platelet activation, we examined the involvement of HK isoforms in glucose metabolism in more detail by performing a glycolytic stress test (Figure 3). Stimulation with convulxin and thrombin caused a rapid increase in platelet glycolysis compared to basal (Figures 3C and 3F), consistent with an elevated glycoATP production rate (Figure 2C). Upregulation of glycolysis by convulxin or thrombin was significantly blocked by 2DG (Figures 3A-C) and 3BP (Figures 3D-F). Taken together, these results suggest that convulxin- and thrombin-induced platelet activation upregulates glucose metabolism in platelets in a HK-dependent manner.

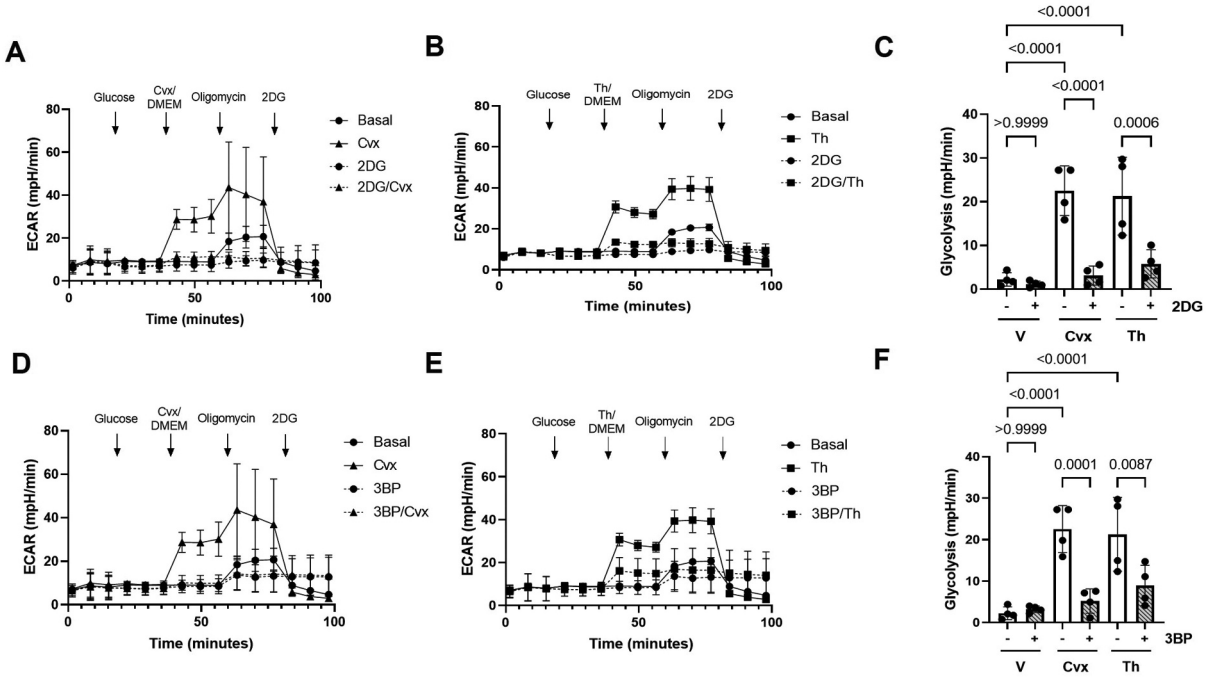

Figure 3. Effects of platelet agonists on glycolytic flux. (A-B) Extracellular acidification rate (ECAR) in WP pre-treated with vehicle or 2DG (50 mM) and stimulated with convulxin (Cvx) (A) or thrombin (Th) (B). (C) Quantification of glycolysis from ECAR measurements ($n = 4$). (D-F) As in (A-C) except WP pre-treated with 3BP (30 μM) ($n = 4$). Data represent mean ± SD. Statistical analysis was performed using one-way ANOVA.

Hexokinase regulates platelet aggregation and reactive oxygen species generation

Having established that 2DG and 3BP inhibit the activation of platelet HK and glycolysis, we examined their effects on platelet aggregation. The concentrations of 2DG and 3BP were first titrated in aggregation assays, stimulated by either convulxin (50 ng/ml) or thrombin (0.03 U/ml) (Supplementary Figures 2A-2B). These experiments identified 2DG (50 mM) and 3BP (30 μM) as concentrations which significantly inhibited the agonist-induced aggregation, without inducing platelet activation and cytotoxicity (Figures 5A–5F). Preincubation of washed platelets with 2DG caused a significant inhibition of convulxin-induced (50 ng/ml) aggregation from 86.5 ± 5.1% to 30.7 ± 14.3% ($p = .0009$; Figure 4A), with similar effects observed with 3BP where aggregation was significantly reduced from 87.7 ± 3.0% to 37.1 ± 25.2% ($p = .0155$; Figure 4B). When we tested the inhibitors against thrombin (0.03 U/ml), both inhibitors significantly reduced platelet aggregation but to a lesser degree with aggregation being reduced from 83.1 ± 6.9% to 53.9 ± 27.6% ($p = .0143$) and from 82.8 ± 7.8% to 53.3 ± 24.3% ($p = .0405$) for 2DG and 3BP, respectively (Figures 4A and 4B). Interestingly, when higher concentrations of agonists were used, the inhibitory effect of HK blockade was overcome during thrombin-induced aggregation but persisted in convulxin-induced aggregation (Supplementary Figure 2C), suggesting agonist-specific differences in the metabolic dependence of platelet aggregation.

Reactive oxygen species (ROS) generation plays a key role in platelet activation.[30] Convulxin-induced robust ROS generation was sensitive to N-acetylcysteine (NAC), whereas thrombin was not (Supplementary Figure 3). Convulxin-induced ROS production was significantly reduced by 2DG (Figure 4Ci) and abolished by 3BP (Figure 4Cii), implicating HK-dependent metabolism in ROS generation.

Hexokinase regulates platelet activation and pro-coagulant platelet formation

Next, we used a multiparameter flow cytometry approach to understand if distinct platelet functions were differentially influenced by HK inhibitors, measuring fibrinogen receptor $\alpha_{IIb}\beta_3$ activation (PAC-1), α-granule release (CD62P), dense granule release (CD63, CD40L), and the constitutive CD42b (GPIβ) expression. As expected, convulxin induced significant increases in all surface markers of activation. The

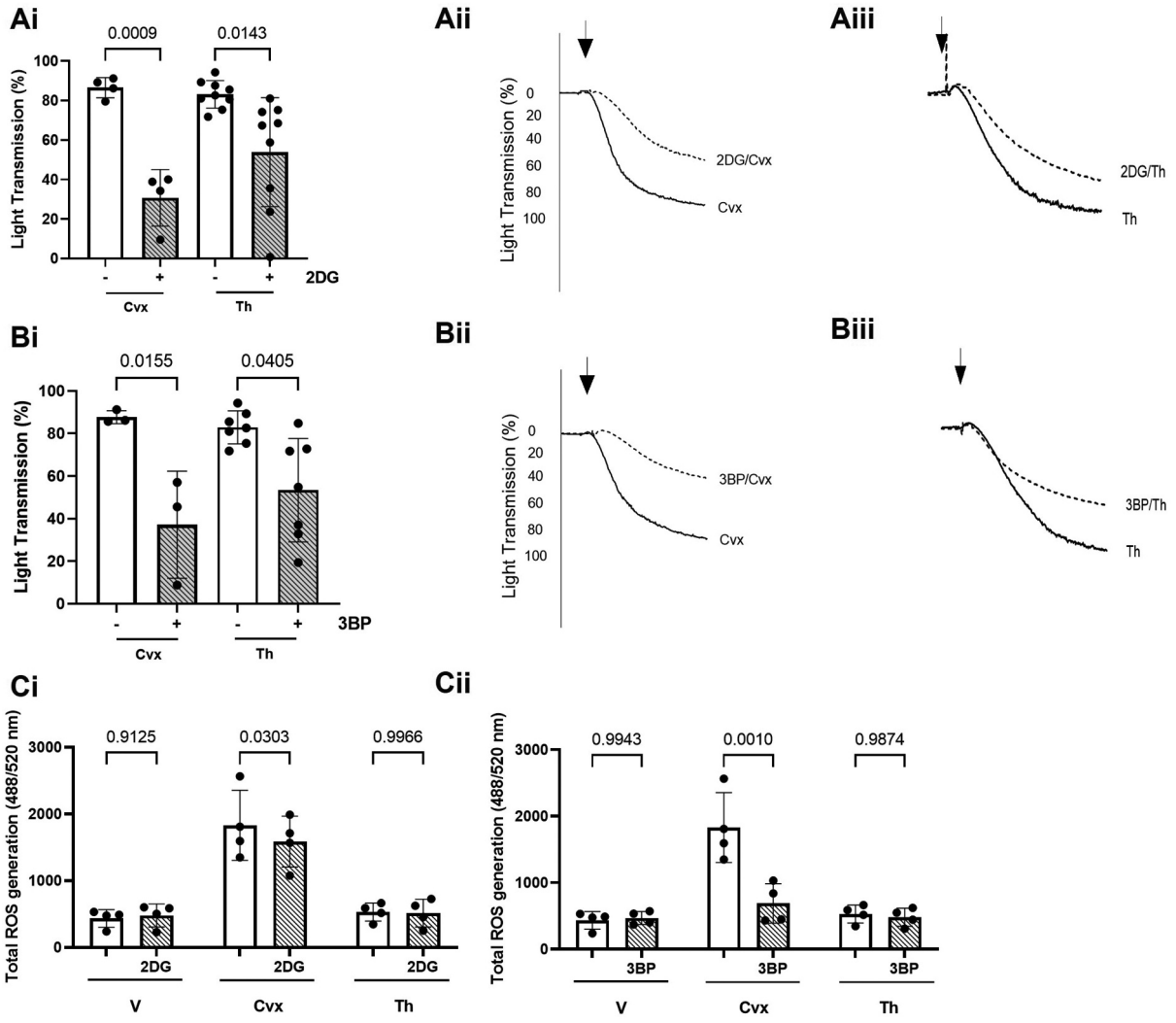

Figure 4. Hexokinase regulates platelet aggregation and ROS generation. (A-C) Platelet aggregation and ROS generation in WP pre-treated with vehicle or 2DG (50 mM) (A) or 3BP (30 μM) (B) and stimulated with convulxin (Cvx, 50 ng/ml) or thrombin (Th, 0.03 U/ml). Aggregation quantification (Ai and Bi) and representative traces (Aii-iii) are shown ($n = 3–9$). ROS quantifications are shown in Ci and Cii ($n = 4$). Data represent mean ± SD. Statistical analysis was performed using one-way ANOVA or two-way ANOVA.

presence of 2DG and 3BP led to complete inhibition of agonist-induced integrin activation (Figure 5A). In contrast, CD62P (Figure 5B), CD63 (Figure 5C), and CD40L (Figure 5D) expression were significantly, but not fully reduced to basal levels by 2DG, whereas 3BP reduced all markers to those observed at basal levels (Figure 5). The reduced expression of CD42b on convulxin or thrombin stimulation was recovered by pre-treatment of 2DG and 3BP (Figure 5E). Interestingly, very similar results were observed following thrombin stimulation despite only modest effects of these inhibitors on aggregation (Figures 4A and 4B). At basal, platelets pre-treated with 2DG and 3BP did not induce phosphatidylserine (PS) externalization (Figure 5F). However, when platelets were dual-stimulated with convulxin and thrombin which lead to the formation of pro-coagulant platelets,[31,32] as evidenced by annexin V binding, the increase in PS exposure was abolished by both 2DG and 3BP (Figure 5F). Overall, these data showed that HK activity plays a key role in facilitating multiple platelet functions.

Effect of hexokinase in platelet spreading and in vitro thrombosis

To assess the role of HK in outside-in signaling, platelet adhesion and spreading over fibrinogen were examined (Figure 6A). HK inhibition with 2DG or 3BP did not affect platelet adhesion (Figure 6Bi). In

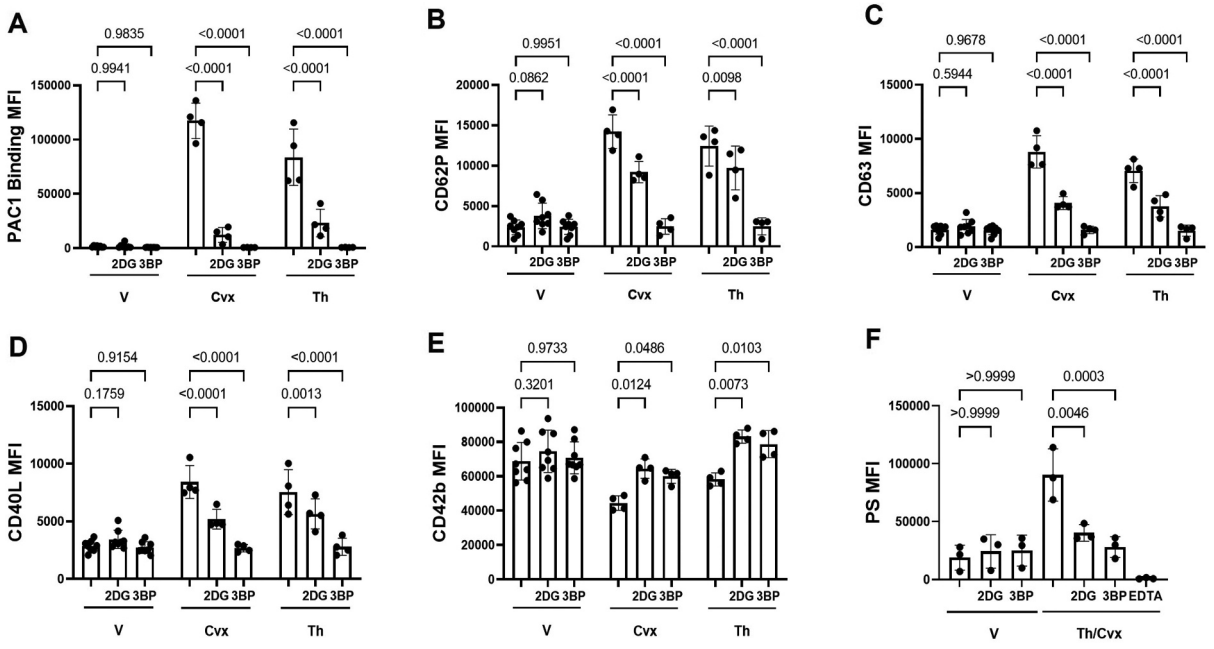

Figure 5. Hexokinase regulates platelet activation and pro-coagulant platelet formation. (A-E) Flow cytometric analysis of platelet activation markers, including PAC1 binding (A), CD62p (B), CD63 (C), CD40L (D), and CD42b (E), in WP pre-treated with vehicle, 2DG (50 mM), or 3BP (30 μM) and stimulated with Cvx or Th ($n = 4–8$). (F) Phosphatidylserine (PS) exposure in WP (under the same conditions; EDTA was used as a negative control) ($n = 3$). Data represent mean ± SD. Statistical analysis was performed using one-way ANOVA or two-way ANOVA.

contrast, platelet spreading was significantly reduced by both inhibitors, with a more pronounced effect observed with 3BP compared to 2DG (Figure 6Bii). Specifically, spreading decreased from 6.06% in vehicle-treated platelets to 4.97% with 2DG ($p = .0225$) and to 2.76% with 3BP ($p < .0001$). These findings suggest that there is a more substantial contribution of HK2 to the regulation of platelet outside-in signaling.

We next sought to determine the physiological relevance of platelet HK activity by examining whole-blood thrombus formation *in vitro* (Figure 6C). Perfusion of vehicle-treated blood over immobilized collagen resulted in robust platelet adhesion and stable thrombus formation (Figure 6C-D). Pre-treatment of the blood with 2DG reduced surface coverage compared to the vehicle control (Figures 6Di-Dii), whereas 3BP showed a non-significant effect ($p = .0529$). Following PBS perfusion, stable thrombus coverage was significantly reduced by both 2DG (4.96 ± 3.30%) and 3BP (6.05 ± 5.23%) compared to the vehicle control (12.69 ± 6.04%) (Figure 6Diii).

Hexokinase plays a key role in hemostasis

To assess if HK is involved in primary hemostasis, we performed a tail-clip assay to measure the bleeding time in C57BL/6 mice. The presence of HK1 and HK2 was first confirmed by immunoblotting (Supplementary Figure 4). The mice were injected intravenously with saline or 2DG (1 mg/g body weight)[21] and rested for 15 minutes before initiation of a tail-clip assay. We found that the 2DG-treated mice had a significantly prolonged bleeding time from 91.8 ± 15.2 seconds for the saline group to 444.8 ± 164.6 seconds (Figure 6Ei). This was consistent with the amount of blood loss, as was measured by hemoglobin accumulation in the buffer compared to control mice, which was significantly increased (Figure 6Eii).

Discussion

Platelet activation requires a dramatic increase in ATP.[10–12] While resting platelets primarily rely on mitochondrial metabolism to meet the basal energy demands,[25] our data demonstrate that activation

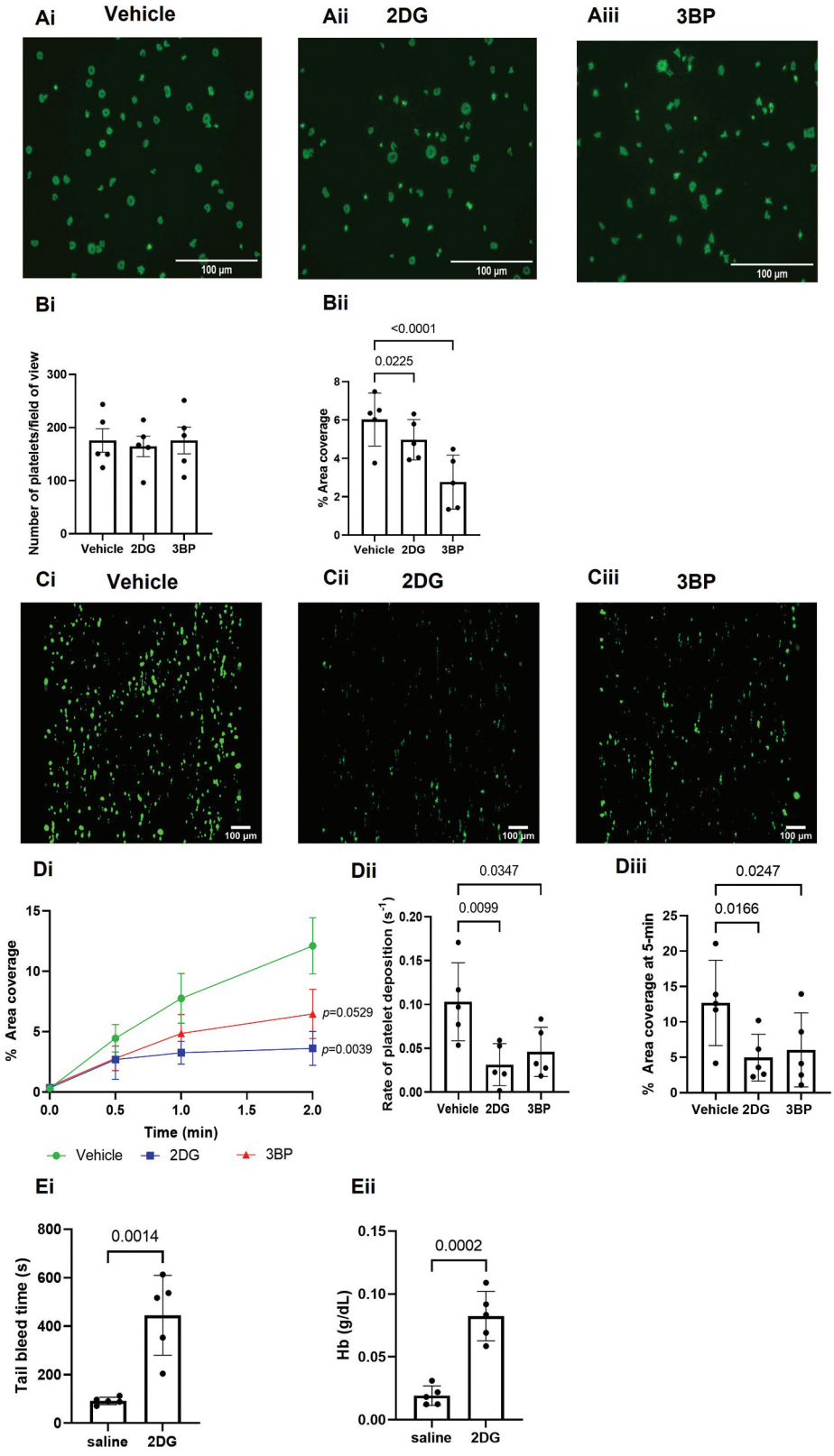

Figure 6. Effect of hexokinase in spreading, *in vitro* thrombosis, and *in vivo* hemostasis. (A) Platelet adhesion and spreading of WP pre-treated with vehicle or 2DG (50 mM) or 3BP (30 µM) before added to fibrinogen-coated wells. Representative images (A) and quantification of the number of platelets/field of view (Bi) and percentage area coverage (Bii) are shown ($n = 4$). (C) *In vitro* thrombus formation under flow in whole blood pre-treated with vehicle, 2DG (50 mM) or 3BP (30 µM) and perfused over collagen (50 µg/ml) coated microfluidic chips at 1000 s^{-1} for 2 minutes. Representative images of platelet adhesion and thrombus formation are shown. (D) Quantification of platelet deposition, including surface coverage (Di), rate of deposition (Dii) and stable adhesion (Diii) ($n = 5$). (E) *In vivo* hemostasis assessed by tail-bleeding assay in mice administered saline or 2DG. Bleeding time (Ei) and hemoglobin loss (Eii) are shown ($n = 5$ per group). Data represent mean ± SD. Statistical analysis was performed using one-way ANOVA, two-way ANOVA, or unpaired t-test with Welch's correction.

induces a shift toward a highly glycolytic phenotype. This metabolic reprogramming, characterized by increased glycolytic flux, highlights a dominant role for glycolysis in supporting platelet activation.

To define the mechanisms underlying this shift, we examined the role of HK, the first rate-limiting enzyme in glucose metabolism. Using pharmacological inhibition, we demonstrate that (i) human and murine platelets express both isoforms of HK, HK1 and HK2; (ii) platelet stimulation leads to the activation of HK; (iii) both HK1 and HK2 contribute to agonist-induced glucose uptake, glycoATP production rate, and glycolysis; (iv) HK activity contributes to platelet aggregation and ROS generation; (v) platelet secretion and outside-in signaling has a greater reliance of HK2; and (vi) HK activity is required for hemostatic function *in vitro* and *in vivo*. Our findings extend early observations in HK-deficient platelets,[33] by showing that acute inhibition of HK activity in normal platelets impairs multiple aspects of platelet functions. These data establish HK as a central regulator of platelet bioenergetics and activation.

We and others have shown that ATP generation from glycolysis is critical for platelet activation.[8,13–15,20,34,35] Most knowledge focuses on thrombin-mediated platelet activation, with little understanding of how glucose metabolism affects specific platelet functions, whether different agonists selectively engage glycolytic pathways, or which signaling nodes mediate crosstalk between platelet activation and metabolism. Fidler *et al.* showed that platelet glucose uptake relies on glucose transporters GLUT1 and GLUT3,[22,36] with GLUT3 mainly stored in α granule[14,37,38] and translocated to the surface upon activation to meet energy demands.[37] Post-uptake, glucose is phosphorylated to glucose-6-phosphate (G-6-P) by HK, maintaining the gradient for continued uptake. Humans express four active HK isoforms, HK1-4, and were observed that two isoforms HK1 and HK were expressed in platelets. We confirmed that the activity of these HKs is critical to maintaining the supply of glucose required for activation. This upregulated HK activity post-platelet activation likely represents an integrated system linked to the increased GLUT3 expression at the platelet surface, providing capacity for glucose influx[22,37] and HK-mediated phosphorylation to maintain the concentration gradient and drive further uptake.[22] Bioenergetic analysis confirmed that ATP generation upon platelet activation is predominantly glycolytic, reflecting the need for rapid ATP generation, but could also point to the requirement to function under variable oxygen conditions within a thrombus. There was also a modest increase in mito-ATP production rate without an increase in oxygen consumption, suggesting enhanced mitochondrial efficiency rather than increased respiratory flux. As expected, inhibition of both HK isoforms reduced both glycoATP production and glycolysis to basal levels.

Consistent with its effect on glycoATP production, both inhibitors abolished agonist-induced glycolysis. Therefore, these data indicate that glucose metabolism during platelet activation relies on HK activity, with HK isoforms functioning in a largely redundant manner.

Previous studies, including ours, show that thrombin-mediated platelet aggregation depends on glycolysis[8,13–15] but can be supported by alternative fuels.[13] Our data with HK inhibitors confirm this metabolic plasticity, since concentrations that fully block HK activity only modestly reduce overall platelet aggregation. We provide evidence that individual hexokinase isoforms potentially regulate distinct platelet functions. Inhibition of all HKs or HK2 alone reduced integrin activation and PS exposure, while in contrast platelet granule secretion (CD62P, CD40L, and CD63) and platelet spreading over fibrinogen were particularly sensitive to HK2 inhibition, suggesting that platelet granule secretion and outside-in signaling may rely on HK2-linked pathways. What is clear was that HK inhibition reduced thrombus formation *in vitro* and prolonged bleeding time in mice, despite short-term 2DG exposure being insufficient to cause thrombocytopenia.[39] Interestingly, while 2DG did not affect mitoATP generation, 3BP reduced it to basal levels. This could suggest a potential role of HK2 in regulating mitochondrial function,[40] possibly through its association with mitochondrial membranes.[41,42] However, this data and the suggestion of isoform-specific effects on platelet functions should be interpreted with caution since 3BP can also affect the activity of pyruvate dehydrogenase[43] and lactate dehydrogenase.[44] This highlights the need for new pharmacological tools including platelet-specific HK isoform inhibitors. The lack of HK1-specific inhibitors and the off-target effects of 3BP mean that future studies using platelet-specific HK1/2 mutant mice or targeted protein degradation are needed to clarify isoform-specific roles. Overall, our data demonstrate that HK activity is essential for thrombus formation and hemostasis, but nonselective inhibition highlights challenges for metabolic targeting.

Our findings complement emerging evidence that platelet activation is regulated by multiple metabolic checkpoints, including cytosolic pyruvate kinase M2 (PKM2) and mitochondrial pyruvate dehydrogenase

kinases (PDKs), which collectively shift metabolism toward glycolysis. Nayak *et al.* reported that activation increases dimeric PKM2 formation,[20] and pharmacological or genetic deletion of PKM2 in mice impairs, but does not abolish, platelet activation, aggregation, clot retraction, and arterial thrombosis, whereas hemostasis remains unaffected. Platelet activation also stimulates PDK, phosphorylating and inhibiting pyruvate dehydrogenase[34,35] to divert pyruvate flux toward glycolysis. PDK inhibition suppresses glucose uptake, aggregation, ATP secretion, and thromboxane A2 (TxA_2) generation, impairing *in vitro* and *in vivo* thrombosis without affecting hemostasis.[34] Whole-body PDK2/PKDK4 single and double knockout studies confirm a predominant role for PDK4 in platelet secretion and thrombosis.[35] These observations resonate with our data on HK isoforms, suggesting a prominent role for HK2 in granule secretion. Together, these pathways suggest that platelet metabolic rewiring occurs at multiple levels to meet energy demands during activation.

Targeting such metabolic nodes may provide an alternative strategy to conventional anti-platelet therapies, potentially overcoming redundancy in receptor-mediated signaling pathways. Although much of the current work exploring this possibility is based in genetically modified mice or the use of untargeted pharmacological agents, there remains the possibility of developing therapies that regulate thrombosis without compromising hemostasis, as has been shown for PDK isoforms.[34,35] However, our data indicate that global inhibition of HK using 2DG markedly impairs hemostasis, emphasizing the need for platelet-specific approaches. In that regard, our work raises questions about the precise roles of HK1 and HK2 in platelet function, and in particular whether they contribute to distinct platelet functions that could guide the repurposing of currently available metabolic inhibitors, such as 2DG and 3BP,[28,41,42,45,46] while carefully considering their systemic effects.

In conclusion, our study identifies HK as a key regulator of platelet metabolism and function. Platelet activation is characterized by enhanced glucose uptake and glycolysis in a HK-mediated mechanism. Blocking HK activity impairs multiple aspects of platelet activation and thrombus formation. These findings highlight HK as a potential metabolic target for controlling pathological platelet activation.

Author contributions

CRediT: **Lih T. Cheah:** Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing; **Jawad S. Khalil:** Formal analysis, Investigation; **Beth A. Webb:** Formal analysis, Investigation; **Daisie M. Yates:** Formal analysis, Investigation; **Reem Alotaibi:** Formal analysis, Investigation; **Mary McKay:** Formal analysis, Investigation; **Mohammad Ali:** Formal analysis, Investigation; **Thomas Palmer-Dench:** Formal analysis, Investigation; **Emily Horner:** Formal analysis, Investigation; **Mugthalana Allet:** Formal analysis, Investigation; **Fraser Macrae:** Funding acquisition, Investigation, Writing – review & editing; **Cedric Duval:** Investigation, Writing – review & editing; **Amanda J. Unsworth:** Investigation, Writing – review & editing; **Mark T. Kearney:** Funding acquisition, Project administration, Writing – review & editing; **Khalid M. Naseem:** Funding acquisition, Project administration, Writing – review & editing.

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