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Glucose metabolism and metabolic flexibility in blood platelets

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ESSENTIALS

- The metabolic integration processes required for platelet activation are unclear.
- The metabolic plasticity of human platelets are investigated
- Activated platelets exhibit a glycolytic phenotype while preserving mitochondrial function

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- Platelets can switch freely between glucose/glycogen and fatty acids to support aggregation

SUMMARY

Background. Platelet activation is an energy-dependent process, but the type and integrated use of metabolic fuels required to drive activation remains unclear.

Objective. To dissect the metabolic fuel and pathway plasticity required for platelet activation.

Methods. Platelet Oxygen Consumption rate (OCR) and Extracellular Acidification Rate (ECAR) were measured as markers of oxidative phosphorylation (OXPHOS) and glycolysis respectively. Glucose and glycogen were quantified by enzyme-coupled fluorometric assay.

Results: Blood platelets switch freely between glycolysis and OXPHOS using either glucose or fatty acids at rest. The transition of platelets from quiescent to activated state promotes a rapid uptake of exogenous of glucose associated with a shift to a predominant glycolytic phenotype coupled with a minor rise in mitochondrial oxygen consumption. Consistent with this metabolic plasticity, under nutrient limiting conditions, platelets utilised glucose, glycogen or fatty acids independently to support activation. Importantly, the glycolytic switch occurred even in the absence of extracellular glucose, originating from endogenous glycogen. Focusing on the relative flexibility of mitochondrial fuel oxidation of glucose and fatty acids, we found that inhibition of a single fuel oxidation was compensated by the increased oxidation of the other, but when oxidation is inhibited, glycolysis is upregulated. Glutamine had little contribution to mitochondrial oxygen consumption. Analysis of the platelet functional dependency on ATP from different pathways demonstrated that inhibition of both fuel oxidation and glycolysis were required to prevent agonist driven platelet activation.

Conclusion: Platelets have significant metabolic fuel and pathway flexibility, but preferentially use glycolysis for ATP generation when activated.

Keywords: Glycolysis; oxidative phosphorylation; Fatty acids; Glycogen; Blood Platelets.

INTRODUCTION

Blood platelets play a critical role in the cessation of bleeding upon vascular injury. Exposure of sub endothelial matrix proteins at areas of vessel wall damage triggers platelet activation, adhesion and aggregation. The subsequent release of soluble platelet agonists triggers platelet aggregation to ensure rapid haemostasis. Conversely, the inappropriate activation of platelets can promote vascular inflammation, atherogenesis and arterial thrombosis (1,2). The transition of platelets from a quiescent to an activated state requires dramatic changes in the availability of ATP (3–7). Early studies used 6-^{14}C-glucose to confirm that platelets possess the molecular machinery necessary to generate ATP through both glycolysis and oxidative phosphorylation (OXPHOS) of glucose (8,9). These early data have now been confirmed by modern metabolic analysis (10) which has provided an important framework of understanding of platelet metabolism. While platelets express glucose transporters and glucose is an established metabolic substrate, platelets also have the capacity to take up and oxidise fatty acids (11) and glutamine as sources of ATP generation at rest and in response to activatory stimuli (10). Despite this, knowledge of platelet metabolism remains incomplete, since the contribution of internal stores of glycogen has been overlooked. This knowledge is crucial since regulation of metabolic pathways is influenced by the relative abundance of glucose, fatty acids (FA) glutamine and internal stores of energy, and the ability of cells to access, and switch between, these substrates. Thus, while platelets have the potential for metabolic flexibility, we have only a partial understanding of fuel choices by

platelets and whether these are functionally specific. Similarly while platelet mitochondrial dysfunction has been reported in a number of disorders including type-2-diabetes (12,13), sickle cell disease and sepsis (14,15), the overall importance to, and flexibility of, mitochondria in platelet function remains poorly understood. To address this knowledge gap, we have examined if platelet activation was associated with regulated changes in fuel metabolism, which in turn were linked to specific functional responses. Crucially, we have explored the extent to which endogenous reserves of fuel, in the form of glycogen, contribute to platelet metabolic function.

MATERIALS AND METHODS:

Reagents

Studies were approved by the Hull York Medical School Ethics Committee and were conducted in accordance with the Declaration of Helsinki. Antimycin A from Streptomyces sp. (antimycin A), rotenone, Cell-Tak Cell & Tissue Adhesive was from Scientific Laboratory Supplies Limited (Hessle, UK). Glucose standard 5mM/L was from Analox (Stourbridge, UK). Nicotinamide adenine dinucleotide phosphate (NADP) and Hexokinase/Glucose-6-Phosphate Dehydrogenase was from Roche (West Sussex, UK). Collagen Reagens HORM® Suspension (KRH) is from Takeda Cambridge Limited (Cambridge, United Kingdom). All other chemicals were from Sigma-Aldrich (Dorset, UK).

Platelet isolation and aggregation

Human washed platelets were prepared and aggregation and ATP secretion were tested as described previously (16). For experiments in glucose-restricted media, glucose was excluded from the modified Tyrode's buffer. For metabolic analyses, platelets were prepared as

described previously (17) and then diluted to $1x10^8$ platelets/mL in modified Tyrode's buffer (15mM NaCl, 0.55 mM NaH_2PO_4, 2.7mM KCl, 0.5mM $MgCl_2$ and 3mg/L phenol red).

Glucose consumption by platelets in vitro

Washed platelets ($2.5x10^8$ platelets/mL) were stimulated with thrombin (0.01-0.1U/mL) and aliquots removed at 0, 1, 3, 5, 7 and 9 minutes for glucose analysis using enzyme-coupled fluorometric assay modified from (18). Briefly, the conversion of glucose to phosphoglucono-δlactone results in the production of highly fluorescent NADPH. The change in the fluorescence is proportional to the amount of glucose, which can be detected with a fluorescence microplate reader with excitation/emission of 340/460nm.

Measuring platelet oxygen consumption and glycolytic rate

The XFp format Seahorse extracellular flux analyser (Agilent Seahorse Bioscience, MA, USA) was used to measure platelet oxygen consumption (OCR) and extracellular acidification rate (ECAR). XFp micro-plates were coated with Cell-Tak (17), washed with PBS and dried 30 minutes prior to experiments. Washed platelets ($1x10^8$ platelets/ml; 50µL) were seeded on the plate as described previously (17). The volume of each well volume was adjusted to 180 µL with XFp assay medium. Platelets were stimulated with thrombin (0.01 or 0.1U/mL) in the presence or absence of oligomycin (1µM), FCCP (2µM), antimycin/rotenone (2.5µM), 2-deoxyglucose (50mM), etomoxir (40µM), BPTES (3µM) and UK5099 (2µM) (Figure S1). The results were standardised for µg proteins using Microplate DC™ protein assay. Unless stated otherwise, the Seahorse Glycolytic Stress Test, Mitochondrial Stress Test, Cell Phenotype Test and Mitochondrial Fuel Flexibility Tests were carried out according to manufacturers instructions (Agilent Seahorse Bioscience, MA, USA). % Platelet mitochondrial dependency,

capacity and flexibility for glucose and fatty acid oxidation calculated as recommended by the manufacturer (Agilent Seahorse Bioscience, MA, USA);

Glucose: % Dependency= $\left(\frac{Basal\ OCR - UK5099\ OCR}{Basal\ OCR - Etomoxir + BPTES + UK5099\ OCR}\right)$*100%;

%Capacity= $\left(1 - \frac{Basal\ OCR - BPTES + Etomoxir\ OCR}{Basal\ OCR - Etomoxir + BPTES + UK5099\ OCR}\right)$*100%;

%Flexibility= %Capacity- %Dependency.

Fatty acids: % Dependency= $\left(\frac{Basal\ OCR - Etomoxir\ OCR}{Basal\ OCR - Etomoxir + BPTES + UK5099\ OCR}\right)$*100%;

%Capacity= $\left(1 - \frac{Basal\ OCR - BPTES + UK5099\ OCR}{Basal\ OCR - Etomoxir + BPTES + UK5099\ OCR}\right)$*100%;

%Flexibility= %Capacity- %Dependency.

Glycogen Determination

Platelet glycogen content, at rest and after thrombin stimulation, was determined in glucose depleted Tyrode's buffer using the acid hydrolysis method (19). Briefly, platelet samples were subjected to sonication to release all the glycogen. The supernatant was acid-hydrolysed with 4M HCl, boiled for 90 minutes before being neutralised with 2M Na_2CO_3. The liberated glucose was quantified using enzyme-coupled fluorometric assay.

Statistical analysis

Student T-test and analysis of variance (Anova) followed by either Dunn's multiple comparisons or Holm-Sidak multiple comparison tests. Non-linear regression (dose-response curve) was used to compare the concentration of agonist (EC_{50}) that gives half of the

maximal response. Data presented as mean ± SEM for assays with three technical replicates per biological replicate or mean ± SD for three biological replicates. All statistical analyses were performed using GraphPad Prism. * Represents $p<0.05$, ** $p<0.01$, *** $p<0.001$, ****$p<0.0001$

RESULTS

Thrombin-induced platelet activation stimulates glucose uptake

Thrombin stimulation induced rapid glucose depletion from the platelet containing-medium (Figure 1). Under basal conditions, platelets absorbed glucose at a steady state (0.03±0.03 μmol/min/10^8 cells), which increased 15-fold (0.5±0.2 μmol/min/10^8 cells; $p<0.05$) over the first minute after stimulation with thrombin (0.1U/ml; Figure 1Ai, Aii), before returning to basal levels 3 minutes post stimulation (Figure 1Ai, Aii). Using a concentration of thrombin (0.01U/ml) to induce a threshold aggregation response, we observed biphasic depletion of glucose that was linked to the aggregation response (Figure 1Bi, Bii). The rate of glucose depletion increased 8-fold (0.03±0.004 μmol/min/10^8 to 0.28±0.03 μmols/min/10^8; $p<0.01$) within the first minute and was maintained for up to 5 minutes as aggregation plateaued. Interestingly, glucose uptake then increased again to 0.57±0.06 μmols/min/10^8 ($p<0.01$ compared to basal) as the secondary aggregation response was initiated (Figure 1Bi, Bii). The rate of glucose depletion for the secondary aggregation response (5-7min) was significantly higher ($p<0.05$) than the initial aggregation response (1-3 min). These data suggest that thrombin induced platelet activation stimulates dose-dependent glucose uptake, which coincides with the different phases of the aggregation response (Figure 1A, B). To confirm this link between platelet function and glucose metabolism, we measured ECAR as readout of glycolysis. Thrombin stimulated a glycolysis as evidence by a transient increase in ECAR

($p<0.0001$; Figure 1Ci, Cii). Inhibition of TxA_2 generation by indomethacin caused a modest but significant reduction in ECAR (4.2±0.7 to 3.4±0.6 mpH/min/μg protein; $P<0.05$). In contrast inhibition of ADP signalling with apyrase was without effect. These data suggest that platelets actively take up external glucose in response to physiological stimuli that drive energy-demanding functions.

Thrombin-induced maximal glycolytic capacity requires both PAR1 and PAR4 receptors

We next examined the maximal glycolytic capacity (20) of platelets (maximal rate of conversion of glucose to lactate under stressed conditions), at rest and during activation using the 'XFp glycolytic stress test'. The basal ECAR was increased to 1.63 ± 0.6 mpH/min/μg protein ($p<0.01$) with glucose addition (5.6 mM), giving the basal glycolytic function (Figure 2Ai, Aii). The injection of oligomycin, to inhibit all mitochondrial ATP generation, increased ECAR to 4.0 ± 0.2 mpH/min/μg protein ($p<0.001$), which was taken as maximal glycolysis in resting platelets. The addition of 2-DG ablated the ECAR, indicating that the ECAR was from glycolysis and that non-glycolytic ECAR was negligible (Figure 2Ai, Aii). Stimulation with thrombin (0.1U/mL) (in the presence of glucose) caused a 10-fold increase in ECAR (5.9±0.4mpH/min/μg protein; $p<0.001$) (Figure 2Bi, Bii), which began to fall over the next 25 minutes. The subsequent treatment with oligomycin increased ECAR to 5.43 ± 0.4 mpH/min/μg protein. Since this increase was no different to that induced by thrombin, it suggested that thrombin induced maximal glycolytic capacity. Thrombin activation of platelets requires ligation of both PAR1 and PAR4 (21) and so we next examined their relative contribution to platelet glycolytic capacity (Figure S2A and B). The PAR1 and PAR4 specific peptides induced dose-dependent glycolytic responses (Figure 2C, D). However, neither peptide stimulated ECAR to the same extent as thrombin at maximal doses. Consistent with

these observations, oligomycin was able to increase ECAR further in both PAR1 (5.8±0.1mpH/min/μg protein; $p<0.001$) and PAR4 (4.0±0.1 mpH/min/μg protein; $p<0.001$). These data suggest that platelets have sufficient glycolytic scope to generate ATP in the absence of mitochondrial function, which can be increased significantly by platelet activation.

Platelet activation induces a glycolytic phenotype while preserving mitochondrial function

The inhibition of mitochondrial ATP synthesis with oligomycin increased glycolysis (Figure 2), suggesting that OXPHOS contributes to ATP generation. Therefore, we examined the partitioning of glucose to glycolysis and OXPHOS. To create conditions where OCR and ECAR were mostly glucose-dependent, platelets were treated with etomoxir, an inhibitor of carnitine palmitoyltransferase-1 (10,22), to eliminate the contribution of fatty acid β-oxidation to OCR. When stimulated with thrombin (0.1U/mL), OCR rose from 1.3±0.02 to 2.0±0.03 pmol/min/μg protein ($p<0.01$; Figure 3Ai, Aii). This rise was abolished by the addition of inhibitors of complex I/III of the mitochondrial respiratory chain, antimycin A and rotenone (A/R). This increase in OXPHOS was accompanied by a 4-fold increase in ECAR (1.7±0.1 to 7.1±0.72mpH/min/μg protein; $p<0.01$) (Figure 3Bi, Bii). Next, an energy phenotype experiment (Agilent Seahorse Bioscience, MA, USA) was performed without etomoxir to explore whether metabolic changes were the consequence of the inhibition of endogenous fatty acids oxidation (Figure 3D). Basally, platelets utilise glycolysis and OXPHOS almost equally (Figure 3D). Activation of platelets led to increased activity of both aerobic glycolysis and OXPHOS (Figure 3A, B, D), but critically there was a shift to a glycolysis- dominated phenotype (almost 3 fold change in ECAR) where oxidative phosphorylation changed only minimally (Figures 3C and S2B).

Endogenous reserves can fuel platelet activation

After establishing the importance of glucose metabolism in platelets, the role of endogenous fuels in the absence of exogenous glucose was explored. The omission of glucose from the medium, containing no other energy substrates, had no effect on thrombin induced platelet aggregation (Figure 4Ai) or ATP secretion (Figure 4Aii) or collagen-induced aggregation and secretion (Figure S2C), but was accompanied by a significant decrease in intracellular glycogen (23.5±4.3 to 10.6±3.5 μg/10^8 cell) ($p<0.05$; Figure 4B). Since externally sourced glucose was not a prerequisite for platelet activation, the relationship between exogenous glucose and endogenous glycogen in thrombin-stimulated platelets was explored (Figure 4Ci). In the absence of external sources of energy, thrombin increased ECAR (0.7±0.1 to a maximum of 4.6±0.4mpH/min/μg protein; $p< 0.001$), which was unaffected by oligomycin. In contrast, when replete with exogenous glucose platelets were able to respond to oligomycin by increasing ECAR (Figure 4Cii). Given that these data suggested that glycogen could support platelet function but require exogenous glucose to sustain glycolysis, the metabolic fate of glycogen upon platelet activation was explored. In contrast to experiments performed in the presence of external glucose (Figure 3Ai), the omission of extracellular glucose, inhibition of β-oxidation caused a sustained and significant fall in OCR (Figure 4Di), suggesting that in the absence of exogenous glucose, endogenous fatty acids are critical to maintaining basal OXPHOS. Nevertheless, thrombin (0.1U/mL) increased OCR by 2.5 fold (2.1±0.1 pmol/min/μg protein; $p<0.01$) and ECAR by 10-fold (4.6±1.3mpH/min/μg protein; $p<0.01$; Figure 4D). Thus, when platelets are deprived of extracellular glucose, thrombin still provokes a switch to a glycolytic phenotype with the preserved mitochondrial function and that glycogen shares similar metabolic fate as glucose.

Platelet mitochondrial fuel flexibility

Having established that platelets can take up glucose, but readily utilise endogenous glycogen and fatty acids in the absence of glucose, the relative flexibility of platelets in oxidising glucose, endogenous fatty acids and glutamine were explored. **Flexibility** is defined as the difference between the **capacity** that exists for a given pathway to increase to a maximal and the absolute minimum **dependency** on that pathway. We first demonstrated that glutamine contributed minimally to mitochondrial oxygen consumption (Figure S3A), leaving glucose and FA as the main oxidative fuels. Thrombin-induced aggregation was unchanged by the UK5099 (an inhibitor of glucose oxidation) or etomoxir when used alone or in combination (Figure 5A), indicative of metabolic plasticity. Next, the platelet flexibility to oxidise glucose at rest and after activation were investigated measuring OCR (Figure 5) and ECAR (Figure S3). UK5099 reduced basal OCR by 36.0± 3.5% (Figure 5Bi) indicating platelet dependency on glucose oxidation. However, there is the capacity for this to increase to 58.3±2.1% when fatty acid and glutamine oxidation are inhibited (Figure 5Bii). The difference between the full capacity and dependency is equivalent to the platelets flexibility on glucose oxidation, 22.2±0.6% of total OCR (Figure 5Biii). By contrast, activation by thrombin (0.1U/mL) increased platelet dependency on glucose oxidation, accounting for 48.6±3.8% of total OCR (Figure 5Ci). Overall capacity to oxidise glucose did not change from that seen in resting platelets (54.3±1.5% of total OCR; Figure 5Cii and Ciii) indicating that activation is supported by platelets' ability to utilize metabolic scope.

Next the platelet flexibility to oxidise endogenous fatty acids was measured at rest and after activation. Inhibition of fatty acid oxidation by etomoxir reduced platelet OCR by 30.63±7.9% (Figure 5Di). The subsequent inhibition of glucose and glutamine oxidation indicated that full

capacity of platelets to oxidise fatty acids was 56.0±7.4% of total OCR (Figure 5Dii). These data indicate that platelets can increase the proportion of OCR dedicated to fatty acid oxidation by 25.4±6.9% in the absence of other fuels (Figure 5Diii). Thrombin (0.1U/mL) did not have significant effect on platelet dependency (37.7±4.0%), capacity (54.3±1.5%) and flexibility (23.2±0.5%) on fatty acid oxidation (Figure 5E) compared to unstimulated platelets. This may be due to the limited pool of metabolically available endogenous fatty acids. The inhibition of fatty acid and glucose/glutamine oxidation pathways independently, did not affect ECAR. However, inhibiting all major fuel oxidation increased ECAR significantly ($p<0.05$; SF 3B, C). These data indicate that inhibition of a single fuel oxidation can be compensated by the increased oxidation of the other. Critically when all the fuel oxidation is inhibited, glycolysis is upregulated for the compensation of energy generation.

Mitochondrial function of resting and activated platelets

The fuel flexibility of platelets prompted the investigation of platelet functional dependency on ATP from glycolysis and mitochondrial OXPHOS. The individual inhibition of total mitochondrial ATP synthesis with oligomycin, or glucose metabolism with 2-DG did not affect platelet aggregation. However, almost total inhibition of platelet aggregation was achieved in combination (Figure 6Ai, Aii) suggesting that platelets have sufficient glycolysis to support the need for ATP even when mitochondrial ATP machinery is manipulated. Having established this flexibility, we next performed a detailed analysis of mitochondrial components of respiration in resting platelets. Platelet mitochondrial components of respiration were measured with the sequential injection of oligomycin, to provide the OCR linked to ATP synthesis, FCCP, a mitochondrial uncoupler, which reveals maximal respiration, and a combination of antimycin A and rotenone (Figure 6Bi). ATP-linked OCR, non-

mitochondrial OCR, proton leak and reserve respiratory capacity were 76.6±5.0%, 11.6±7.0%, 11.7±6.0% and 34.5± 7.0%, respectively (Figure 6Bii). Thrombin (0.1U/mL) increased OCR from 2.1±0.2 to 3.1±0.4 pmol/min/µg protein ($p<0.001$; Figure 6Biii, Biv), which was associated with increased ATP-linked OXPHOS, increased maximal respiration and reserve capacity. Inhibition of platelet derived TxA_2 generation by indomethacin (10µM) significantly reduced thrombin-induced OCR, ATP-linked OCR, maximal respiration and reserve capacity (all $p<0.001$ compared to thrombin alone). The omission of glucose did not significantly alter any of the parameters (Figure 6D).

Thus, platelets use their reserve respiratory capacity in addition to increasing glycolysis to satisfy the increased need for ATP synthesis in response to thrombin stimulation, which requires platelet-derived TxA_2 to support mitochondrial function (Figure 6C). Furthermore, glycogen alone can fully support the mitochondrial function during platelet activation (Figure 6D).

Sf 4b

DISCUSSION

Our knowledge of the metabolic fuel choices and how key metabolic pathways are regulated to support platelet functions are unclear. In the present study, we demonstrate that platelets use both glucose/glycogen and endogenous FA to support glycolysis and OXPHOS as means of maintaining baseline ATP demand. Upon platelet activation, the cells adopt a glycolytic phenotype regardless of the nutrient availability. However, this change to a glycolytic state during cellular activation is underpinned by a remarkable metabolic versatility, whereby under normal physiological conditions, platelets are able to move freely between mitochondrial glucose and FA oxidation.

Glucose is an abundant, readily available nutrient for platelets *in situ*, yet our current understanding of platelet glucose metabolism is limited in terms of the functional requirements for glucose and the glycolytic capacity of platelets under stressed conditions. The abundance of GLUT3 suggests that a sustained glucose transport into platelets is guaranteed even in a low glucose environment, and is indirectly indicative of the importance of glucose to platelet function, both at rest and following activation (23–25). Recent studies have now demonstrate that the loss of GLUT3 can fundamentally diminish key elements of platelet function (26). Thus, focusing on carbohydrate metabolism, we found that platelet glucose uptake was potentially linked to the phase of activation in response to external stimuli. Consistent with this observation, thrombin stimulation caused a receptor mediated significant increase in glycolysis. The maximal glycolytic rate of resting platelets was less than that of thrombin-stimulated, which required both PAR1 and PAR4 receptors (Figure 1-2). Activatory cells have a metabolic 'capacity', which enables them to respond quantitatively and sensitively to changing energy demands, despite the fact that under normal conditions, they operate at a lower metabolic rate (27). Platelets clearly display this type of glycolytic capacity, as glycolysis increases in response to the inhibition of OXPHOS, suggesting a Pasteur effect-type of metabolic compensation, and that platelets utilise this 'capacity' to support activation.

Next, we demonstrated a clearer understanding of the biological role of glycogen, the interplay between glycogen and exogenous glucose and their metabolic fate in platelets. We found that glycogen alone can support platelet activation and that it shared similar metabolic fate with glucose. Focusing on the partitioning of glucose and glycogen in glycolysis and OXPHOS, or data confirm previous studies that the pathways of glycolysis and glucose oxidation are present in platelets (10,28–30). In addition to this, the current study has reconfirmed that activated platelets adopt a glycolytic phenotype while preserving the

mitochondrial function, regardless of fuel availability. Importantly, our data has established for the first time that this phenotype occurs in the absence of externally supplied glucose, indicating that endogenous reserves can serve as a fuel source (Figure 3-4). These data suggest that the functional responses were driven by choosing aerobic glycolysis over OXPHOS of glucose and glycogen consistent with a 'Warburg' or aerobic glycolytic phenotype (31,32). The commitment to a less efficient energy-generating pathway as glycolysis is not unusual and has been reported in granulocytes, dendritic cells (33), monocytes (34) and macrophages (35). Thus, a glycolytic switch upon stimulation observed in blood platelets may represent a conserved immunometabolic pattern in developmentally related cells of blood lineages. While many nucleated immune cells switch to Warburg metabolism to support proliferation (36), it is likely that the low efficiency but rapid rate of ATP production by glycolysis is required to support a rapid change in energy demand in response to platelet activation (37). The preserved mitochondrial function while presenting a glycolytic phenotype suggests an ATP requirement as well as redox metabolism beyond glycolysis to maintain cell survival (38–40).

Metabolism of glucose, fatty acids and amino acids converge in mitochondria, where fuel oxidation can be modified in response to changes in the availability of nutrients and the energy demand of the cell under various conditions (41). Focusing on platelet mitochondrial function (Figure 5-6), we show that platelets can switch freely between glucose and fatty acid oxidation both at rest and under activated conditions. Earlier studies suggest that fatty acid β-oxidation may compensate for a reduction of glycolysis (42). However, we show that when oxidation of glucose and fatty acids are restricted, platelets up-regulate aerobic glycolysis to meet the energy demand for functional responses, indicating not only fuel, but metabolic pathway flexibility in order to respond to a thrombotic signal. The majority of oxygen

consumed by platelets is linked to ATP synthesis, with only modest contributions from proton leak and non-mitochondrial functions. Platelet activation leads to increased oxidative metabolism facilitated by the presence of a significant mitochondrial 'reserve capacity', which provides additional scope for mitochondrial function necessary for responding to increases in ATP demand in times of work or stress (43). Interestingly, maximal thrombin induced OCR required the release of platelet derived TxA_2, suggesting that agents secondary to the initial platelet activation is required to promote maximal OXPHOS. The omission of glucose did not affect mitochondrial function, again highlighting the metabolic plasticity of platelets. This presents the possibility that platelets possess uniqueness in terms of metabolic flexibility when compared to other immune cells. For example, the inhibition of fatty acid oxidation blocked T cell differentiation into T_{reg} whereas supplementation with fatty acids supported its function (44). Similarly, the polarization of M_2 macrophages is fatty acid dependent and the inhibition of fatty acid oxidation drives M_2 macrophages to M1 state (45). Mature dendritic cells have both OXPHOS and aerobic glycolysis where the inhibition of glycolysis prevents dendritic cell maturation (46). For platelets, such flexibility may be important to ensure function in a range of environments including within the thrombus, where the availability of nutrients and oxygen may be limited (47,48). The availability of endogenous glycogen, fatty acids and the flexibility to produce sufficient ATP solely from glycolysis or OXPHOS in platelets indicate a potential survival mechanism for the most adverse microenvironments (34,49) Alternatively, it is possible that flux and use of specific metabolic pathways are linked to distinct functions. Detailed bioenergetic profiling of platelet immune and angiogenic functions will be required to fully appreciate how the metabolic microenvironment including availability of oxygen and nutrients determine the influence of platelets on multiple aspects of vascular biology.

In summary, this study describes a detailed systematic analysis of fuel choice made by human platelets at rest and under activating conditions. Of note, platelets adopt a glycolytic phenotype when stimulated with thrombin regardless of the nutrient availability and thrombin dose. Most importantly, this study describes a remarkable metabolic plasticity, switching freely between substrates and metabolic pathways, of blood platelets under physiological conditions. A better understanding of platelet metabolism and modulation of fuel availability may offer new opportunities to optimise long-term storage conditions. Moreover, there is increasing evidence showing that transition between resting to activated state of the activatory cells require specific metabolic patterns (36,50) to support functional changes (10,34,51). In this context, improving our understanding of platelet metabolism and its regulation could lead to the development of novel therapeutic targets for treating arterial thrombosis and potentially other inflammatory diseases.

Addendum

M. Aibibula designed & performed experiments, analysed the data and drafted the manuscript. K.M. Naseem designed the research and wrote the manuscript. R. Sturmey designed the experiment and the research and wrote the manuscript.

Disclosure of Conflict of Interest

The authors state that they have no conflict of interest.

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Figure Legends

FIGURE 1. Thrombin-induced platelet activation stimulates glucose uptake.

(A) Human washed platelets ($3x10^8$ platelets/ml) were stimulated with thrombin (0.1U/ml) followed by measurement of aggregation and glucose depletion from the medium. (i) Representative aggregation trace of three independent experiments. (ii) Glucose depletion rate from the medium over a 9 minutes period after thrombin stimulation. Results were expressed as rate of glucose depletion (μmols/min/10^8 platelets), mean ± SEM, n=3. **(B)** As in (A) except platelets were stimulated with thrombin (0.01U/ml). **(C)** The Extracellular Acidification Rate (ECAR) was measured for platelets treated with thrombin (0.1U/ml), in the presence and absence of apyrase (2U/ml) and indomethacin (10μM). (i) Average ECAR plotted over time. (ii) Average ECAR from C(i) with data presented as mean ± SEM, n=3. *$p<0.05$; **$p<0.01$; ****$p<0.0001$

FIGURE 2. Thrombin-induced maximal glycolytic capacity requires both PAR1 and PAR4 receptors

(A) ECAR of human platelets was measured in Glycolytic Stress Test with sequential injection of glucose (5.6mM), oligomycin (1μM) and 2-deoxy glucose (2-DG, 50mM). (i) Average ECAR plotted over time. (ii) Average ECAR quantified from A(i). The average was taken after each injection. **(B)** As in (A) except platelets were stimulated with thrombin (0.1U/ml). **(C)** ECAR of human platelets was measured with 2.5, 5, and 10μM of PAR1. (i) Average ECAR plotted over time. (ii) Average ECAR from C(i). **(D)** ECAR of human platelets was measured with 10, 20,

and 50μM of PAR4. (i) Average ECAR plotted over time. (ii) Average ECAR from D(i). All data presented as mean ± SEM, n=3. *p<0.05; **p<0.01; ***p<0.001

FIGURE 3. Platelet activation induces a glycolytic phenotype while preserving mitochondrial function

(A) Oxygen Consumption Rate (OCR) was measured with sequential injection of etomoxir (40μM), thrombin (0.1/0.01 U/ml), antimycin A & rotenone (A/R; 2.5 μM) and 2-DG. (i) Average OCR of platelets plotted over time. The glucose/glycogen dependent oxygen consumption was separated by the addition of etomoxir. Non-mitochondrial OCR was accounted by the OCR insensitive to a combination of A/R. (ii) Average OCR quantified from A(i). **(B)** ECAR of platelets measured simultaneously with (A). 2-DG was added to correct for non-glycolytic extracellular acidification. **(C)** Fold changes in glucose-dependent OCR & ECAR over basal was quantified from (A) and (B). The proportions are calculated for the values that were corrected for non-mitochondrial OCR & non-glycolytic ECAR. **(D)** XFp Cell Phenotype Test was carried out in the absence of etomoxir. The ratio between OCR and ECAR was plotted under basal and stressed conditions. Stressed OCR and ECAR were measured with combination of oligomycin and FCCP in the presence of 0.1U/ml thrombin. All data shown as mean ± SEM, n=3. **p<0.01

FIGURE 4. Endogenous reserves can fuel platelet activation

(A) Human washed platelet aggregation and ATP secretion was tested in the presence and absence (+/-) of glucose in the media. (i) Dose response curve of platelets stimulated with thrombin in +/- glucose media. EC_{50} was compared for each dose with data shown as mean

± SD, n=3. **(B)** Platelet glycogen level before after thrombin (0.1U/ml) stimulation in glucose-free media. Data shown as mean ± SD. **(C)** ECAR of human platelets was measured in Glycolytic Stress Test with thrombin (0.1U/ml) in the presence or absence of glucose. (i) Average ECAR plotted over time with sequential injection of 0.1U/ml thrombin, oligomycin and 2-DG in glucose-free media. (ii) Average EACR plotted over time with sequential injection of 0.1U/ml thrombin, glucose, oligomycin and 2-DG in glucose-free media. **(D)** OCR was measured in glucose-free media with sequential injection of etomoxir, thrombin (0.1 U/ml), A/R and 2-DG. (i) Average OCR plotted over time. (ii) Average OCR quantified from D(i). (iii) Average ECAR measured simultaneously with D(i) and plotted over time. (iv) Average ECAR quantified from D(iii). (v) Fold changes in glycogen-dependent OCR & ECAR over basal was quantified from D(i) and D(iii). The proportions are calculated for the values that were corrected for non-mitochondrial OCR & non-glycolytic ECAR. Data expressed as mean ± SEM, n=3. *p<0.05; **p<0.01; ***p<0.001

FIGURE 5. Platelet mitochondrial fuel flexibility

(A) Human washed platelets were stimulated with thrombin (0.1U/ml) followed by measurement of aggregation in the presence of etomoxir and UK5099 (2μM). (i) Representative aggregation trace of three independent experiments. (ii) % Platelet aggregation was quantified from A(i) with data expressed as mean ± SD, n=3. **(B)** Platelet mitochondrial flexibility for glucose oxidation was measured using XFp Mitochondrial Fuel Flexibility Test. (i) Average OCR was plotted over time for platelet dependency to oxidise glucose. Basal OCR was established before the sequential injection of UK5099 and the combination of etomoxir and BPTES. (ii) Average OCR was plotted over time for platelet capacity to oxidise glucose. Basal OCR was established before the sequential injection of the

combination of etomoxir and BPTES followed by UK5099. (iii) % Platelet mitochondrial dependency, capacity and flexibility for glucose oxidation calculated from B(i) and B(ii). The average was taken after each injection. **(C)** As in (B) except platelets were stimulated with thrombin (0.1U/ml). **(D)** Platelet mitochondrial flexibility for fatty acid oxidation was measured using XFp Mitochondrial Fuel Flexibility Test. (i) Average OCR was plotted over time for platelet dependency to oxidise fatty acids. Basal OCR was established before the sequential addition of etomoxir and the combination of UK5099 and BPTES. (ii) Average OCR was plotted over time for platelet capacity to oxidise fatty acids. Basal OCR was established before the sequential addition of the combination of UK5099 and BPTES followed by etomoxir. (iii) % Platelet mitochondrial dependency, capacity and flexibility for endogenous fatty acid oxidation calculated from D(i) and D(ii). The average was taken after each injection. **(E)** As in (D) except platelets were stimulated with thrombin (0.1U/ml). Data shown as mean ± SEM, n=3.

FIGURE 6. Mitochondrial function of resting and activated platelets

(A) Human washed platelets were stimulated with thrombin (0.1U/ml) followed by measurement of aggregation in the presence of oligomycin and 2-DG. (i) Representative aggregation trace of three independent experiments. (ii) % Platelet aggregation was quantified from A (i) with data expressed as mean ± SD, n=3. **(B)** Platelet mitochondrial function was measured using XFp Mitochondrial Stress Test. (i) Average OCR was plotted over time for platelet with sequential injection of oligomycin, FCCP and antimycin & rotenone (A/R). (ii) ATP-linked OCR, non-mitochondrial OCR, proton leak and reserve capacity was calculated as % of basal OCR from B(i). (iii) As in B(i) except platelets were stimulated with thrombin (0.01U/ml). (iv) Average OCR quantified from B(iii).The average was taken after

each injection. **(C)** As in B(iii) except platelets were treated with Indomethacin (10μM). **(D)** As in B(iii) except for the absence of glucose in the medium. Data shown as mean ± SEM, n=3. *p<0.05; **p<0.01;p<0.001; ****p<0.0001

FIGURE 1

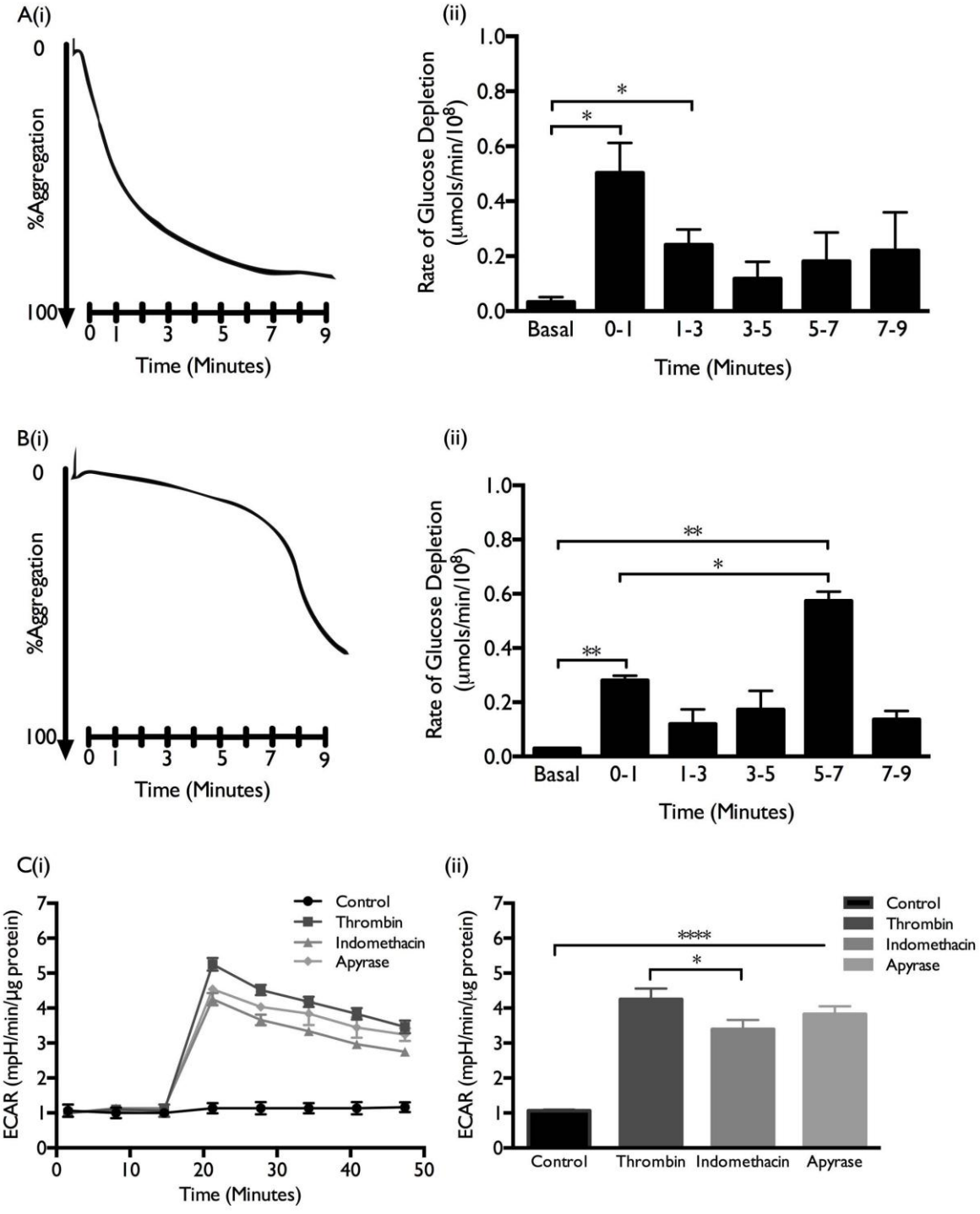

FIGURE2

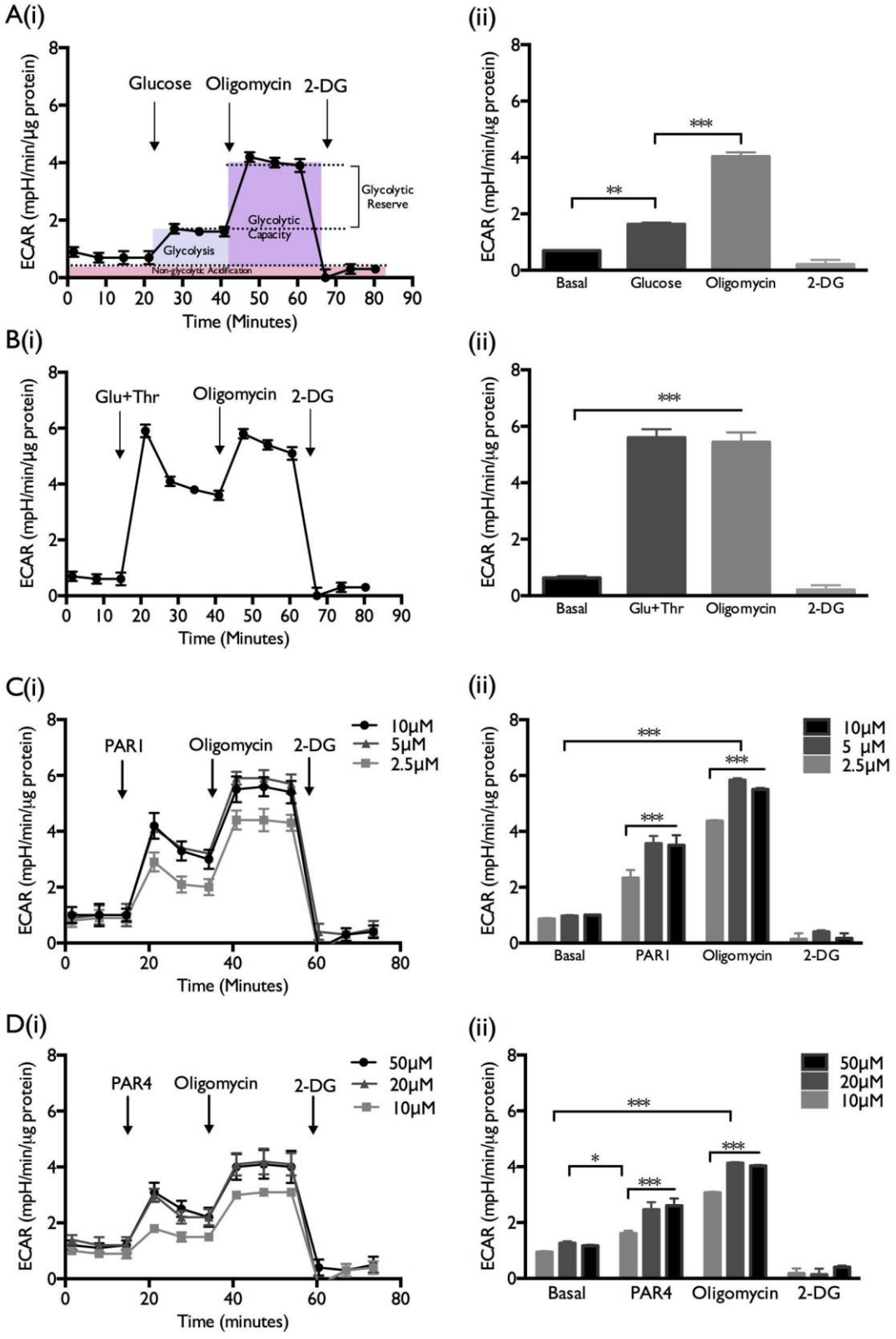

FIGURE3

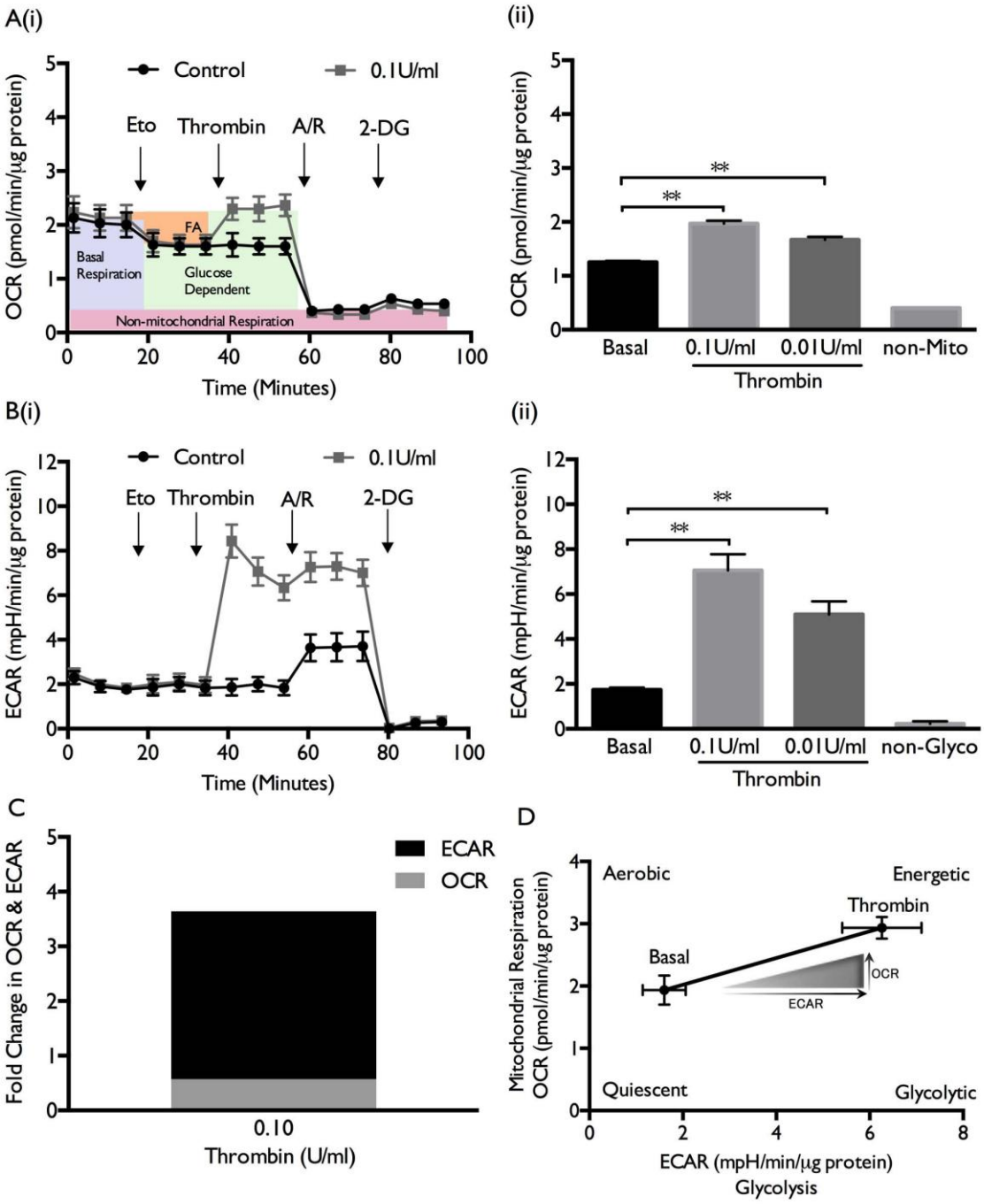

FIGURE4

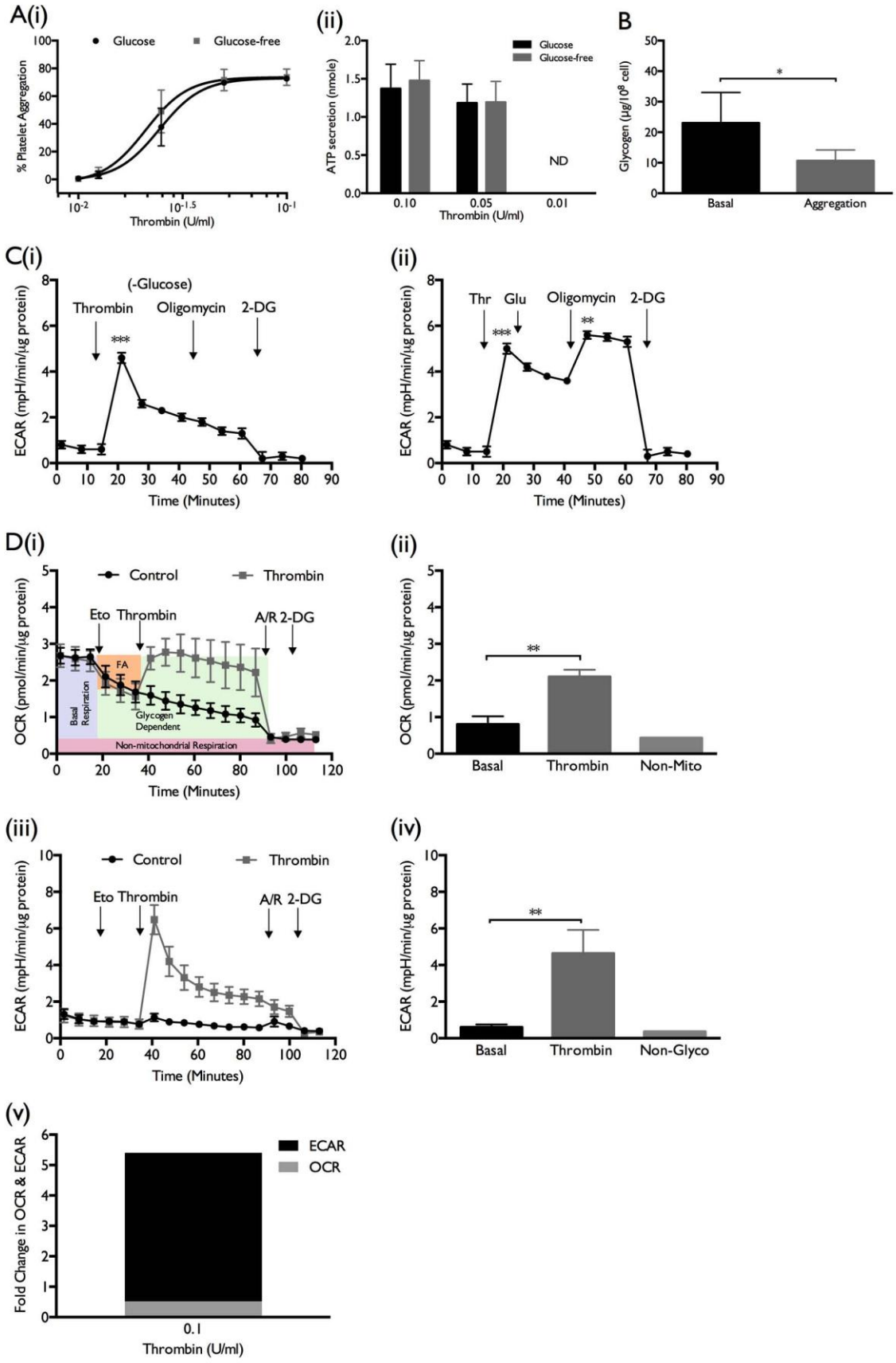

FIGURE 5

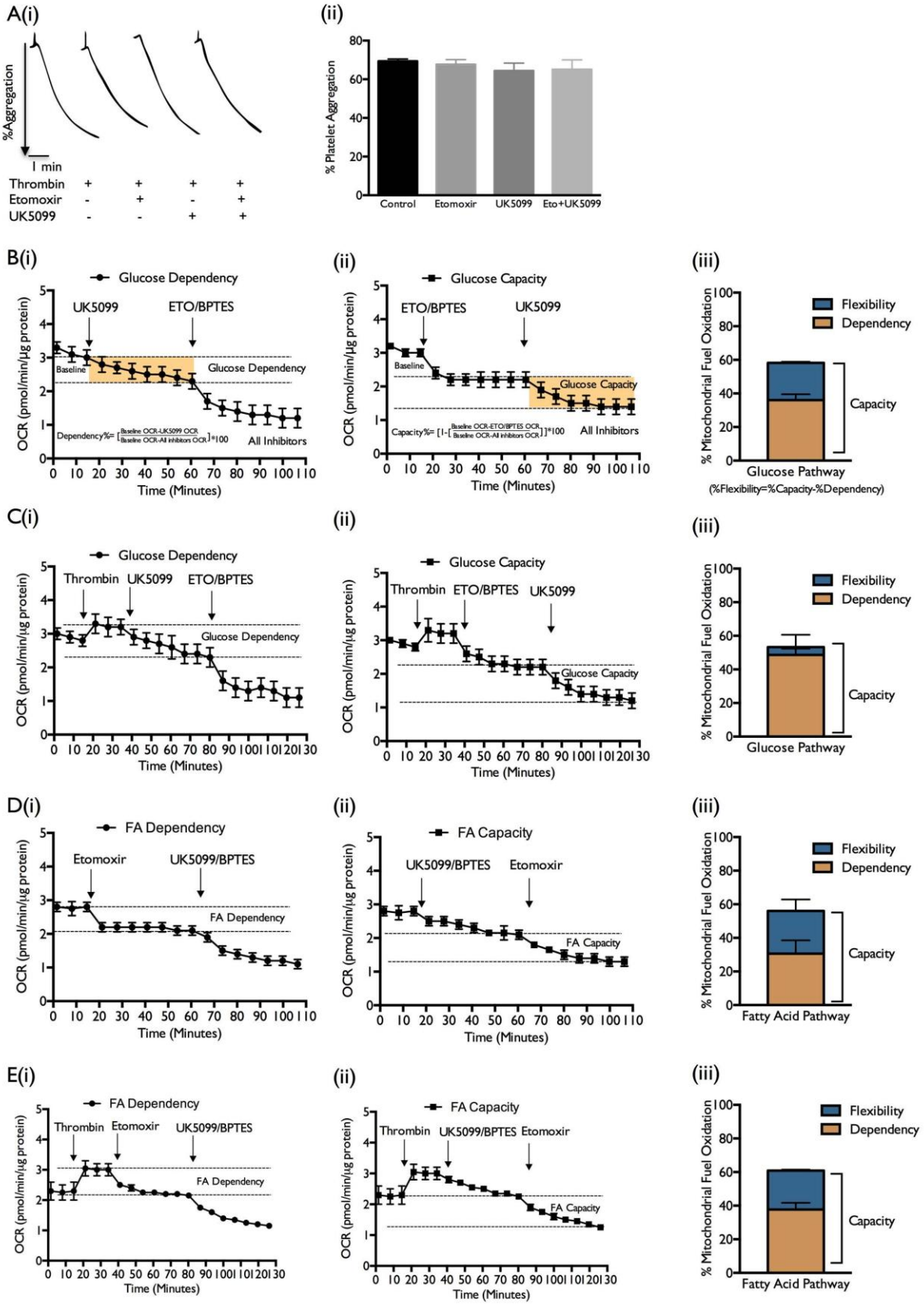

FIGURE6