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## Rapid kinetics of changes in oxygen consumption rate in thrombin-stimulated platelets measured by high-resolution respirometry

Alice P. Sowton <sup>a, b</sup>, Sarah L. Millington-Burgess <sup>a</sup>, Andrew J. Murray <sup>b</sup>,  
Matthew T. Harper <sup>a, \*</sup><sup>a</sup> Department of Pharmacology, University of Cambridge, UK<sup>b</sup> Department of Physiology, Development and Neuroscience, University of Cambridge, UK

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## ABSTRACT

Platelet activation plays a key role in normal haemostasis and pathological thrombosis. Platelet activation is rapid; within minutes of stimulation, platelets generate bioactive phospholipids, secrete their granule contents, activate integrins and aggregate together to form a haemostatic plug. These events are dependent on ATP synthesis. Mitochondrial function in platelets from healthy volunteers and patients with a range of diseases indicate an important role for oxygen consumption in oxidative phosphorylation in normal and pathological function. Platelets also consume oxygen during oxidation reactions, such as cyclooxygenase-dependent thromboxane A<sub>2</sub> synthesis. In this study, we used high-resolution respirometry to investigate rapid changes in oxygen consumption during platelet activation. We demonstrated a rapid, transient increase in oxygen consumption rate within minutes of platelet stimulation by the physiological activator, thrombin. This was partly inhibited by aspirin and by oligomycin. This shows that high resolution respirometry can provide information regarding rapid and dynamic changes in oxygen consumption during platelet activation.

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## 1. Introduction

Platelet activation is central to haemostasis and arterial thrombosis [1]. Platelets adhere at sites of vascular injury, generate and release bioactive phospholipids, release their granule contents through exocytosis, and activate their major integrin,  $\alpha_{IIb}\beta_3$ . These events lead to recruitment of further platelets and platelet aggregation, forming a haemostatic plug or occlusive thrombus [1].

Platelet activation requires ATP, which is provided by increased mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis [2–5]. Pioneering experiments starting in the 1970s using Clark-type oxygen electrodes showed that platelet activation with a range of physiological stimuli triggers a rapid increase in O<sub>2</sub> consumption, attributed to increased mitochondrial OXPHOS (inhibited by oligomycin) and oxidation of arachidonic acid by cyclooxygenase (COX; inhibited by aspirin) [6,7]. However, Clark-type electrodes show a number of problems, including uniform

signal drift, changes in sensor response, and bubble formation on the electrode [8]. Importantly, the resolution achievable with Clark-type electrodes is relatively low compared to high-resolution approaches currently available.

More recent methodologies for studying O<sub>2</sub> consumption, such as the Oxygraph-2k high-resolution respirometer (Oroboros Instruments) and the high-throughput Seahorse Extracellular Flux (XF) Analyzer (Seahorse Bioscience Inc.), have stimulated renewed interest in the regulation of O<sub>2</sub> consumption in many primary cell types. Each system has its own advantages and disadvantages [compared in detail in Ref. [9]]. The Seahorse XF has been used to characterise platelet O<sub>2</sub> consumption, mitochondrial function and glycolysis. This has usually been studied in unstimulated platelets, either from healthy donors [9–11], or from patients with conditions such as sickle cell disease [12], pulmonary hypertension [13], type 2 diabetes [14] or preeclampsia [15]. A small number of studies have reported changes in platelets following stimulation with thrombin, which increased O<sub>2</sub> consumption rate by approximately 25% at the next measured time (approximately 8 min later) [3,16]. By this time, many of the rapid platelet functional responses have already occurred, including granule secretion and integrin activation [17]. Platelet O<sub>2</sub> consumption and mitochondrial function have also been

\* Corresponding author. Department of Pharmacology, Tennis Court Road, Cambridge, CB2 1PD, UK.

E-mail address: [mth29@cam.ac.uk](mailto:mth29@cam.ac.uk) (M.T. Harper).

studied in the Oxygraph-2k. These studies have focused on respiration in unstimulated platelets from healthy donors [18], patients with sepsis [19] or Alzheimer's disease [20], and in response to drug treatment [21,22] or high-intensity training [23]. However, we are not aware of any studies that report the kinetics of rapid changes in  $O_2$  consumption using the Oxygraph-2k system in response to platelet stimulation with physiological activators such as thrombin.

In this study, we used the Oxygraph-2k to investigate changes in platelet  $O_2$  consumption following stimulation by thrombin. We describe a rapid, transient increase in  $O_2$  consumption rate that is partly dependent on COX activity and OXPHOS, but also depends on other pathways. This shows that high-resolution respirometry can provide information regarding rapid and dynamic changes in  $O_2$  consumption during platelet activation.

## 2. Methods

### 2.1. Materials

All reagents were purchased from Sigma unless otherwise specified. Amidated peptides, SFLLRN-NH<sub>2</sub> and AYPGKF-NH<sub>2</sub>, were from Bachem (Weil am Rhein, Germany).

### 2.2. Washed platelet preparation

Blood was drawn by venepuncture into sodium citrate-containing vacutainers (3.8% v/v) from healthy, drug-free volunteers, who had given written, informed consent in accordance with the Declaration of Helsinki. Use of human blood from healthy volunteers was approved by the Human Biology Research Ethics Committee, University of Cambridge. Acid citrate dextrose (85 mM tri-sodium citrate, 71 mM citric acid, 111 mM D-glucose) was added (1:7 v/v) and platelet-rich plasma (PRP) separated by centrifugation (200 g, 10 min, room temp., no brake). Prostaglandin E<sub>1</sub> (100 nM; Cambridge Bioscience) and apyrase (Grade VII; 0.02 U/ml) were added to PRP to prevent platelet activation during preparation. Platelets were pelleted from PRP by centrifugation (600 g, 10 min, room temp., with brake) and resuspended at a density of  $1 \times 10^8$  platelets/ml in HEPES-buffered saline (HBS; 135 mM NaCl, 10 mM HEPES, 3 mM KCl, 1 mM MgCl<sub>2</sub>, 0.34 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, supplemented with 0.9 mg/ml D-glucose). Platelets were rested at 30 °C for 30 min prior to experimentation. For inhibition of COX, platelets were incubated with aspirin (300 μM) 30 min prior to being added to the Oxygraph chamber. Control samples were incubated with an equivalent amount of the vehicle (DMSO) for the same time. In contrast, oligomycin (or its vehicle, ethanol, as control) were added directly to platelets in the Oxygraph chamber, as indicated in the Results section.

### 2.3. Measurement of oxygen flux during platelet activation

Oxygen consumption in washed human platelets was measured using an Oxygraph-2k high-resolution respirometer (Oroboros Instrument, Innsbruck, Austria) in 2 ml glass chambers at a constant temperature of 37 °C and stirrer speed 750 rpm. Oxygen flux ( $JO_2$ ), which is directly proportional to oxygen consumption rate, was continuously recorded with a 2 s sampling rate using DatLab software 6.1 (Oroboros Instruments, Austria). Calibration at air saturation was carried out every day prior to experimentation, and all data were corrected for background instrumental oxygen flux in accordance to the manufacturer's instructions. Platelets ( $1 \times 10^8$ /ml in HBS with glucose, as described above) were added to the Oxygraph chambers and allowed to reach a stable baseline oxygen consumption rate prior to stimulation in the presence of CaCl<sub>2</sub> (2 mM). All reagents injected into the chambers (CaCl<sub>2</sub>, thrombin,

PAR agonists, oligomycin or vehicle control) were warmed to room temperature prior to addition.

### 2.4. Statistical analyses

Data are reported as mean  $\pm$  standard error of the mean (SEM); the reported  $n$  value indicates the number of independent platelet preparations from different donors. Data were analysed by paired  $t$ -test for comparison of two conditions, and by RM two-way ANOVA with Sidak multiple comparison test for comparison of the effect of oligomycin in the absence or presence of aspirin.

## 3. Results

### 3.1. Thrombin triggers a rapid increase in oxygen flux in platelets

Washed platelets were treated with DMSO (0.1%; the vehicle control for aspirin – see next section) then stimulated with the physiological activator, thrombin (1 U/ml) in the presence of extracellular CaCl<sub>2</sub> (2 mM). Extracellular oxygen concentration was monitored. This was converted to the oxygen flux,  $JO_2$ , which is directly proportional to  $O_2$  consumption rate, and is shown in Fig. 1A–B. Prior to stimulation,  $JO_2$  was  $8.5 \pm 0.4$  pmol s<sup>-1</sup> 10<sup>-8</sup> platelets. Thrombin triggered a rapid, transient increase in  $JO_2$ . The peak increase in  $JO_2$  was  $39.6 \pm 5.6$  pmol s<sup>-1</sup> 10<sup>-8</sup> platelets higher than the pre-stimulation baseline (Fig. 1C; a  $5.4 \pm 0.6$ -fold increase) and occurred at  $72.8 \pm 2.9$  s after stimulation (Fig. 1D).  $JO_2$  then decreased over the following 20 min. A previous study using the Seahorse XF [3] reported that thrombin stimulated an increase in oxygen consumption rate by 'approximately 25%' at the next reported point of measurement (8 min later). For comparison, in our experiments, after 10 min stimulation with thrombin,  $JO_2$  was  $38.3 \pm 13.0\%$  higher than the pre-stimulation baseline (Fig. 1E;  $n = 5$ ;  $p = 0.045$ ).

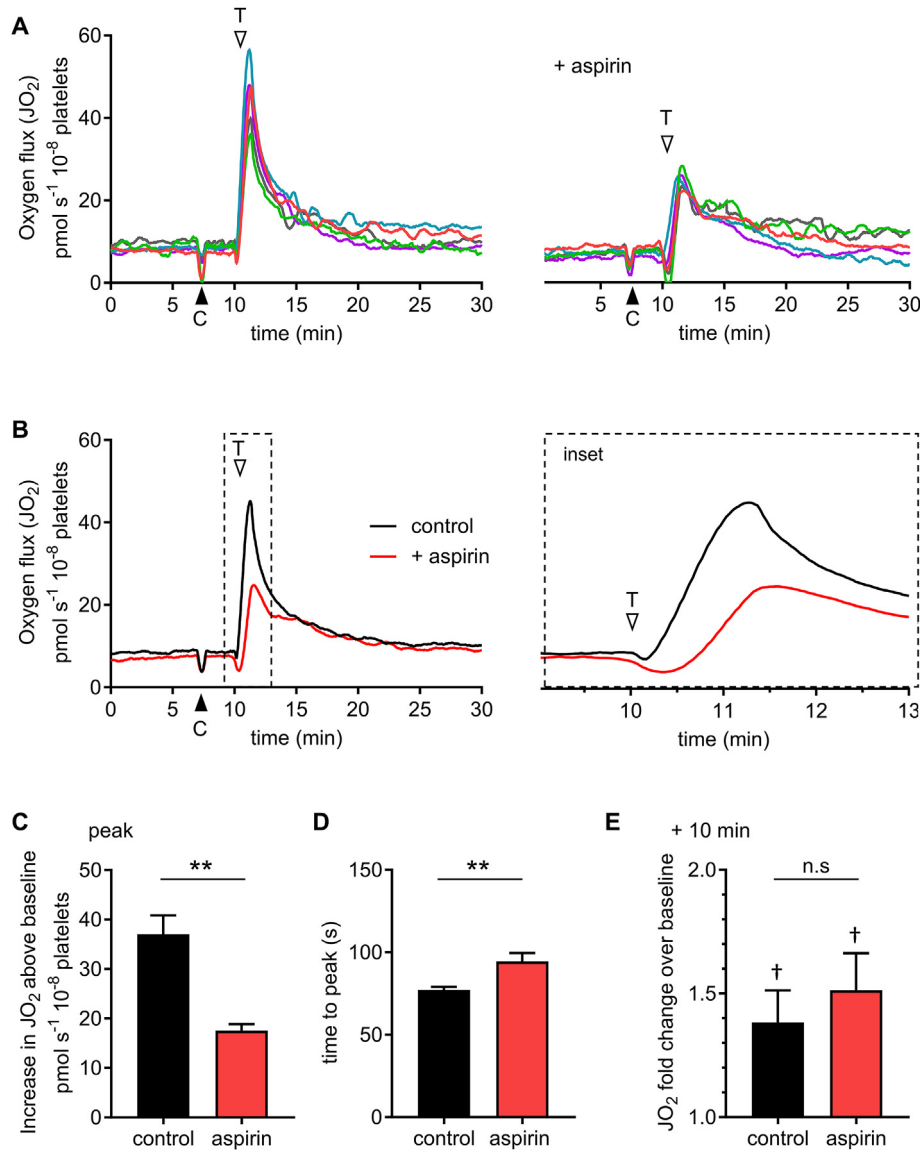
### 3.2. COX activity contributes to the rapid increase in $JO_2$

COX enzymes use oxygen in the generation of eicosanoids from arachidonic acid [24]. Arachidonic acid (500 μM) stimulated a rapid increase in  $JO_2$ , which was inhibited by aspirin, a COX inhibitor (300 μM; Suppl. Fig. 1). To assess the contribution of COX to the thrombin-triggered increase in  $JO_2$ , platelets were stimulated in the presence of aspirin (Fig. 1A, right hand panel). The peak increase in  $JO_2$  was significantly inhibited and delayed by aspirin (Fig. 1C–D). (The delay in reaching peak  $JO_2$  is clearly shown in the inset panel of Fig. 1B.) However, thrombin was still able to trigger an increase in  $JO_2$  that was  $2.4 \pm 0.3$ -fold higher than the pre-stimulation baseline, indicating that thrombin also rapidly activates other sources of  $O_2$  consumption.

In contrast to the peak increase in  $JO_2$ , the increased  $JO_2$  10 min after stimulation was not significantly affected by aspirin (Fig. 1E;  $p = 0.93$ ;  $n = 5$ ).

### 3.3. Protease-activated receptor agonists rapidly increase $JO_2$

In human platelets, thrombin activates the protease-activated receptors, PAR1 and PAR4, and glycoprotein Ib (GPIb). It has been reported that thrombin-stimulated  $O_2$  consumption is dependent on GPIb, and that stimulation of PAR1 does not increase  $O_2$  consumption [25]. However, we observed a rapid and transient increase in  $JO_2$  when platelets were stimulated with SFLLRN-NH<sub>2</sub> (10 μM) or AYPGKF-NH<sub>2</sub> (200 μM), selective agonists of PAR1 and PAR4, respectively (Fig. 2A–B). Under these conditions, aspirin significantly inhibited the peak increase in  $JO_2$  in response to SFLLRN-NH<sub>2</sub>, from  $16.9 \pm 5.9$  to  $6.9 \pm 0.7$  pmol s<sup>-1</sup> 10<sup>-8</sup> platelets



**Fig. 1. Thrombin triggers a rapid increase in oxygen flux ( $JO_2$ ), which is partly inhibited by aspirin.**

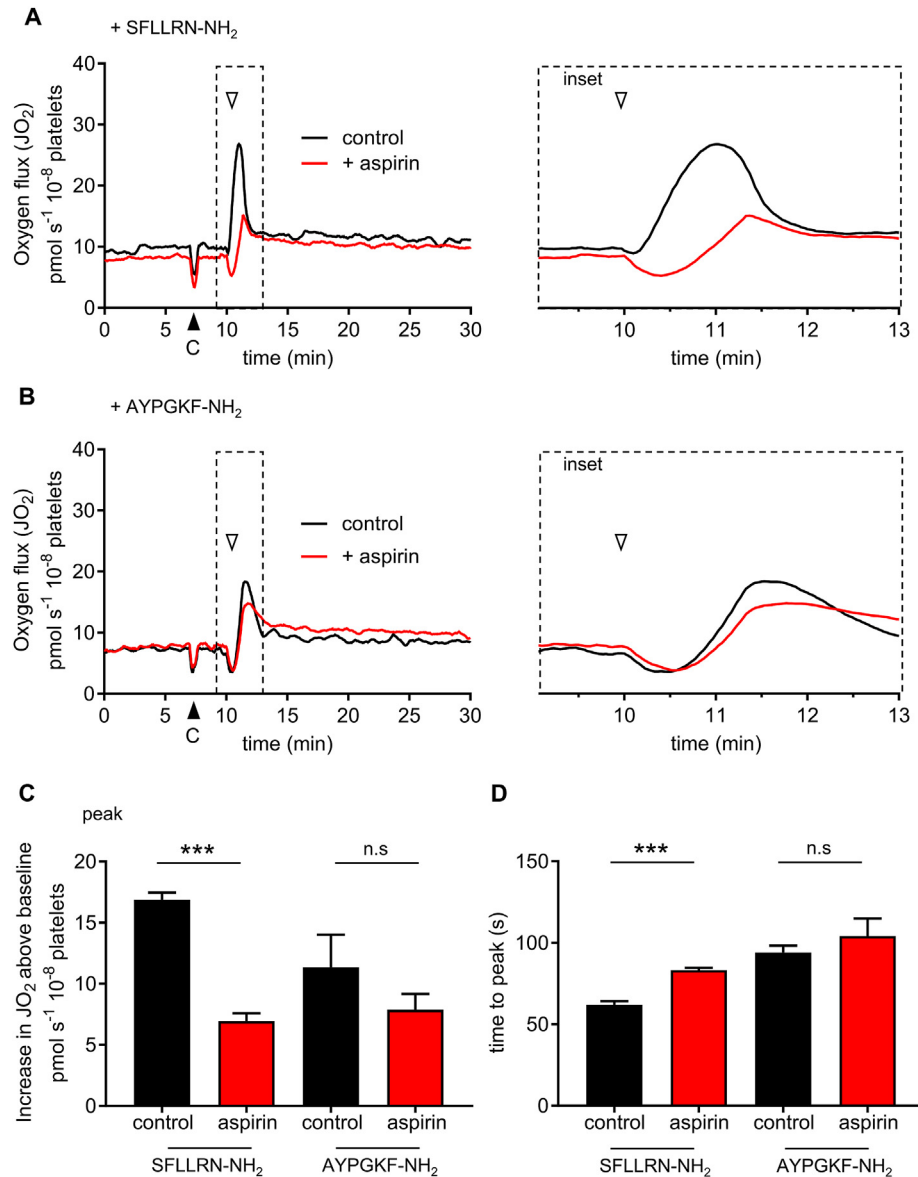
Washed platelets were treated with aspirin (300  $\mu M$ ) or its vehicle (DMSO, 0.1%; 'control') then stimulated with thrombin in an Oxygraph-2k high resolution respirometer.  $CaCl_2$  (final concentration, 2 mM) was added at the black arrowhead ('C'). Thrombin (1 U/ml) was added at the white arrowhead ('T'). In **A**, individual traces from platelet independent platelet preparations (different donors) are shown. The control (DMSO) is the left-hand panel, and aspirin-treated platelets are shown in the right-hand panel. Matched colours indicate the pair experiments. The mean signal ( $n = 5$ ) is shown in **B**; the dashed region is expanded in the right-hand panel. The peak change in  $JO_2$  (above the baseline prior to thrombin addition) is shown in **C** and the time from thrombin addition to this peak is shown in **D** (mean + SEM;  $n = 5$ ; \*\* $p < 0.01$  for control vs. aspirin). The  $JO_2$  10 min after thrombin addition is shown in **E**, reported as fold change over the baseline (n.s. not significant for control vs. aspirin; † $p < 0.05$  for fold-change at 10 min significantly different to pre-stimulation baseline). This measure was chosen to aid comparison with a previous report, as described in the *Results* section.

( $n = 5$ ;  $p < 0.001$ ), but had a less consistent effect when platelets were stimulated with AYPGKF-NH<sub>2</sub> (the peak increase in  $JO_2$  was  $11.3 \pm 2.7\ pmol\ s^{-1}\ 10^{-8}\ platelets$  in the absence of aspirin, and  $7.9 \pm 1.3\ pmol\ s^{-1}\ 10^{-8}\ platelets$  in aspirin-treated platelets;  $n = 5$ ;  $p = 0.22$ ). These data are summarised in Fig. 2B–D. 10 min after stimulation with SFLLRN-NH<sub>2</sub>,  $JO_2$  was  $14.5 \pm 3.9\%$  above the pre-stimulation baseline ( $n = 5$ ;  $p < 0.05$ ), whereas 10 min after AYPGKF-NH<sub>2</sub>,  $JO_2$  was  $16.0 \pm 7.1\%$  ( $n = 5$ ;  $p = 0.85$ ) above the pre-stimulation baseline.

#### 3.4. The higher post-stimulation $JO_2$ is inhibited by oligomycin

To determine the contribution of OXPHOS to the thrombin-stimulated changes in  $JO_2$ , we treated platelets with oligomycin (2.5  $\mu M$ ), an inhibitor of the mitochondrial  $F_1F_0$ -ATP synthase.

Oligomycin rapidly decreased baseline  $JO_2$  (Fig. 3A–B;  $p = 0.011$  compared with vehicle control). The peak increase in  $JO_2$  was also partially inhibited by oligomycin, either in the absence or presence of aspirin (Fig. 3A and C). The increase in  $JO_2$  10 min after stimulation was inhibited by oligomycin in the presence of aspirin (Fig. 3D). By 20 min post-stimulation, aspirin had no effect and oligomycin completely inhibited the remaining increase in  $JO_2$  (note that this is relative to an already-inhibited baseline). This indicates that oligomycin-sensitive OXPHOS contributes to the rapid, transient increase in  $JO_2$ , and to the sustained smaller increase. Notably, other sources of  $O_2$  consumption also contribute to the peak change in  $JO_2$ , as the rapid increase in oxygen consumption was not completely inhibited by aspirin and oligomycin in combination.



**Fig. 2. Protease-activated receptor agonist peptides trigger a rapid increase in JO<sub>2</sub>.**

Washed platelets, treated with aspirin or vehicle control as in Fig. 1, were stimulated with SFLLRN-NH<sub>2</sub>, a PAR1 agonist (A) or AYPGKF-NH<sub>2</sub>, a PAR4 agonist (B). The traces represent the mean signal (n = 5). The black arrowhead indicates addition of CaCl<sub>2</sub>. The white arrowhead indicates addition of agonist peptide. The dashed area is shown expanded as an inset panel to the right. The peak change in JO<sub>2</sub> (above the baseline prior to thrombin addition) is shown in C and the time from agonist peptide addition to this peak is shown in D (mean + SEM; n = 5; \*\*\*p < 0.001 for control vs. aspirin, n.s. not significant; n = 5).

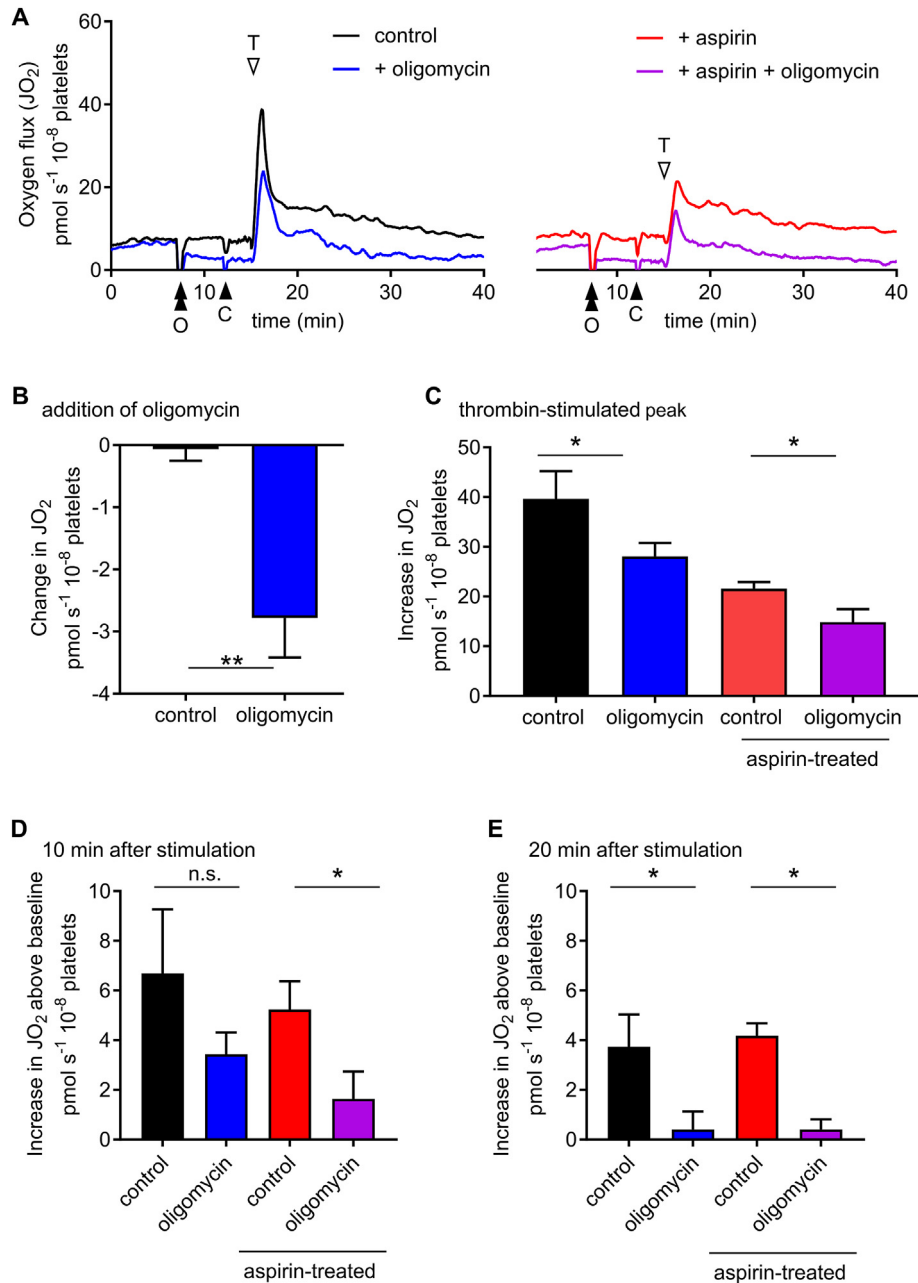
#### 4. Discussion

Platelet stimulation with thrombin triggers a rapid, transient increase in JO<sub>2</sub>. The peak of this increase occurred slightly later than 1 min after stimulation and then declined to a new baseline over the following 10–20 min. The rapid increase in JO<sub>2</sub> occurs on the same timescale as more-commonly measured platelet functions, such as aggregation or granule secretion and shows striking similarities to thrombin-triggered intracellular Ca<sup>2+</sup> signalling [e.g. Ref. [26]]. In contrast, published reports of platelet O<sub>2</sub> consumption using the Seahorse XF have so far missed this rapid increase in JO<sub>2</sub> [3]. Extracellular flux analysis has shown a relatively small increase in oxygen consumption rate (OCR) of around 25%. This is similar in magnitude to the approximately 38% average increase in JO<sub>2</sub> that we observed 10 min after stimulation, after most of the peak increase in JO<sub>2</sub> had subsided. This suggests that although the

published methods using Extracellular Flux Analysis may be useful for investigating more sustained changes in oxygen consumption in platelets following stimulation, high resolution respirometry is more useful for investigating the rapid, transient burst of O<sub>2</sub> consumption that occurs immediately following platelet stimulation.

In this study, the platelets were isolated from plasma and resuspended in HEPES-buffered saline with 5 mM glucose as the exogenous source of metabolic substrates. This is a relatively common approach for many platelet signalling studies [27], and an advantage of this approach is that data can be readily compared with published data on platelet aggregation, granule secretion and intracellular signalling. However, the effect of adding other exogenous substrates, such as glutamine, pyruvate or fatty acids, could be investigated in this protocol, and may affect the extent of increase in OXPHOS following stimulation [3].

The rapid increase in JO<sub>2</sub> in response to thrombin was also



**Fig. 3. Oligomycin inhibits  $JO_2$  in unstimulated and stimulated platelets.**

Washed platelets were treated with oligomycin (2.5  $\mu$ M) or its vehicle (ethanol, 0.05%) as control at the point indicated by the double black arrowhead ('O'). CaCl<sub>2</sub> was then added (black arrowhead, 'C') followed by thrombin (white arrowhead, 'T'). The traces show mean signal (n = 5) from platelets with either DMSO (0.1%; left-hand panel) or aspirin (300  $\mu$ M; right-hand panel). The immediately decrease in  $JO_2$  following addition of oligomycin is shown in C (mean + SEM; n = 5; \*\*p < 0.01). The mean peak increase in  $JO_2$  above baseline prior to thrombin addition (but after oligomycin or its control) is shown in D (mean + SEM; n = 5; \*p < 0.05 for control vs. ethanol). The increases in  $JO_2$  10 or 20 min after thrombin addition are shown in D or E, respectively.

observed when platelets were stimulated with peptide agonists of PAR1 or PAR4. This is not very surprising, since thrombin triggers intracellular signalling in platelets by activating these receptors [28]. The increase in  $JO_2$  10 min after stimulation was statistically significant for PAR1 stimulation, though this was relatively small, and may not be robustly observed under different experimental conditions. This may explain why a previous report found no increase in OXPPOS in response to PAR1 stimulation [25].

The pioneering studies of the 1970s onwards attributed the rapid increase in O<sub>2</sub> consumption rate to COX activity [6,7]. Although we broadly replicated these observations in this study,

our high-resolution respirometry shows a more complex picture. In our study, aspirin significantly inhibited the rapid, peak increase in  $JO_2$ , consistent with the previous reports. However, with high resolution respirometry, we observe that there is still a substantial increase in  $JO_2$ , indicating that there are other mechanisms of O<sub>2</sub> consumption occurring. This demonstrates the benefit of high-resolution respirometry over Clark-type electrodes in understanding the changes in O<sub>2</sub> consumption during platelet activation. Similarly, in the early studies, oligomycin-sensitive OXPPOS accounted for the basal O<sub>2</sub> consumption in unstimulated platelets but did not significantly contribute to the increased O<sub>2</sub>

consumption in thrombin-stimulated platelets. We also demonstrate that the baseline O<sub>2</sub> consumption could be inhibited by oligomycin and likely represents the contribution of OXPHOS to ATP synthesis in unstimulated platelets. However, we also show that oligomycin inhibits the rapid peak increase in JO<sub>2</sub>, independently of COX activity. This might represent a rapid, transient activation of OXPHOS, which might support ATP-dependent events during the earliest stages of platelet activation. It could also represent another pathway of O<sub>2</sub> consumption that is reduced by the inhibition of baseline ATP synthesis. The remaining O<sub>2</sub> consumption, which is not inhibited by aspirin or oligomycin, could involve other O<sub>2</sub> consuming pathways that are known to be activated in platelets, such as 12-lipoxygenase, xanthine oxidase or NADPH oxidase [29–32].

In summary, we show that high-resolution respirometry can be used to investigate the kinetics of changes in O<sub>2</sub> consumption rate in stimulated platelets, across a timescale that is relevant to the rapid activation of platelets. Our data show that OXPHOS is a major contributor to O<sub>2</sub> consumption in unstimulated platelets. Following stimulation, O<sub>2</sub> is rapidly consumed in a COX-dependent manner, as previously reported. However, the high resolution of this approach shows that other pathways of O<sub>2</sub> consumption are also rapidly activated. This gives a more complex picture of the rapid kinetics of changes in O<sub>2</sub> consumption than was possible with Clark electrodes or has been reported with Extracellular Flux Analysis. High resolution respirometry is therefore a useful tool to investigate the rapid kinetics of changes in O<sub>2</sub> consumption during platelet activation.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.bbrc.2018.08.031>.

## Transparency document

Transparency document related to this article can be found online at <https://doi.org/10.1016/j.bbrc.2018.08.031>.

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