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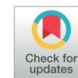
Rapid kinetics of changes in oxygen consumption rate in thrombin-stimulated platelets measured by high-resolution respirometry



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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_2 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_2 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_2 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_2 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_2 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_2 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_2 consumption and mitochondrial function have also been

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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_2 and AYPGKF-NH_2, 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_1 (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 1×10^8 platelets/ml in HEPES-buffered saline (HBS; 135 mM NaCl, 10 mM HEPES, 3 mM KCl, 1 mM $MgCl_2$, 0.34 mM NaH_2PO_4, 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 (1×10^8/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 $CaCl_2$ (2 mM). All reagents injected into the chambers ($CaCl_2$, thrombin, PAR agonists, oligomycin or vehicle control) were warmed to room temperature prior to addition.

2.4. Statistical analyses

Data are reported as mean ± 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 $CaCl_2$ (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 ± 0.4 pmol s^{-1} 10^{-8} platelets. Thrombin triggered a rapid, transient increase in JO_2. The peak increase in JO_2 was 39.6 ± 5.6 pmol s^{-1} 10^{-8} platelets higher than the pre-stimulation baseline (Fig. 1C; a 5.4 ± 0.6 -fold increase) and occurred at 72.8 ± 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 ± 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_2 (10 μM) or AYPGKF-NH_2 (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_2, from 16.9 ± 5.9 to 6.9 ± 0.7 pmol s^{-1} 10^{-8} platelets

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