

Title: A novel flow cytometry assay using dihydroethidium as redox-sensitive probe reveals NADPH oxidase-dependent generation of superoxide anion in human platelets exposed to amyloid peptide β .

Aisha Alsheikh Abubaker¹, Dina Vara², Ian Eggleston¹, Ilaria Canobbio³ and Giordano Pula^{2,*}.

¹: Department of Pharmacy and Pharmacology, University of Bath (UK); ²: Institute of Biomedical & Clinical Science, University of Exeter Medical School (UK); ³: Department of Biology and Biotechnology, University of Pavia (Italy). *: corresponding author.

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Abstract

Reactive oxygen species (ROS) generation is critical in the regulation of platelets, which has important implications in the modulation of haemostasis and thrombosis. Nonetheless, despite several assays have been described and successfully utilised in the past, the analysis of ROS generation in human platelets remains challenging.

Here we show that dihydroethidium (DHE) allows the characterisation of redox responses upon platelet activation by physiological and pathological stimuli. In particular, the flow cytometry assay that we describe here allowed us to confirm that thrombin, collagen-related peptide (CRP) and arachidonic acid but not adenosine diphosphate (ADP) stimulate superoxide anion formation in a concentration-dependent manner. 0.1 unit/ml thrombin, 3 $\mu\text{g/ml}$ CRP and 30 μM arachidonic acid are commonly used to stimulate platelets *in vitro* and here were shown to stimulate a significant increase in superoxide anion formation. The ROS scavenger N-acetylcysteine (NAC) abolished superoxide anion generation in response to all tested stimuli, but the pan-NADPH oxidase (NOX) inhibitor VAS2870 only inhibited superoxide anion formation in response to thrombin and CRP. The involvement of NOXs in thrombin and CRP-dependent responses was confirmed by the inhibition of platelet aggregation induced by these stimuli by VAS2870, while platelet aggregation in response to arachidonic acid was insensitive to this inhibitor.

The pathological platelet stimulus amyloid β ($\text{A}\beta$) 1-42 peptide also stimulated superoxide anion formation in a concentration-dependent manner. $\text{A}\beta$ peptide stimulated superoxide anion formation in a NOX-dependent manner, as proved by the use of VAS2870. $\text{A}\beta$ 1-42 peptide displayed only moderate activity as an aggregation stimulus, but was able to significantly potentiate platelet aggregation in response to submaximal agonists concentrations, such as 0.03 unit/ml thrombin and 10 μM arachidonic acid. The inhibition of NOXs by 10 μM VAS2870 abolished $\text{A}\beta$ -dependent potentiation of platelet aggregation in response to 10 μM arachidonic acid, suggesting that the pro-thrombotic activity of $\text{A}\beta$ peptides depends on NOX activity. Similar experiments could not be performed with thrombin or collagen, as NOXs are required for the signalling induced by these stimuli. These findings shed some new light on the pro-thrombotic activity of $\text{A}\beta$ peptides.

In summary, here we describe a novel and reliable assay for the detection of superoxide anion in human platelets. This is particularly important for the investigation of the pathophysiological role of redox stress in platelets, a field of research of increasing importance, but hindered by the absence of a reliable and easily accessible ROS detection methodology applicable to platelets.

Introduction

The generation of superoxide anion by platelets was described over 40 years ago [1]. In recent years, ROS have been described as critical players in platelet activation both in the healthy body and in disease [2, 3]. A role for ROS and especially superoxide anion in platelet hyperactivation in pathological conditions such as ischemia and hypercholesterolemia has become apparent [4, 5]. Interestingly, scavenging of ROS or inhibition of the enzymatic sources of these highly reactive species has been shown to inhibit platelet activation, which is suggested to underlie the antithrombotic activity of antioxidants [6, 7]. Amongst ROS-generating enzymes, NADPH oxidases (NOXs) play a key role in platelet regulation and haemostasis modulation [8, 9].

Several molecular mechanisms have been described that underly ROS-dependent regulation of platelet activation. These include the increase in ADP release and bioavailability [9], the direct modulation of integrin $\alpha_{IIb}\beta_3$ [10], the modulation of integrin $\alpha_{IIb}\beta_3$ via inhibition of protein phosphatases [11], the reduction of nitric oxide levels by formation peroxynitrite [11, 12], the modulation of GPVI and its signalling [13], or the modulation of protein kinase C (PKC) [14]. How these molecular pathways affect different functional responses, downstream of which agonists they are activated and how they interact with each other is currently under investigation.

Recent studies confirmed the importance of NOXs in the activation of platelets, although there is a discrepancy regarding which NOX enzymes are responsible for platelet activation in response to different stimuli. Human and mouse platelets express both NOX1 and NOXs [15]. ROS generated by NOX1 and NOX2 promote platelet activation via the Syk/phospholipase C γ_2 /calcium signaling pathway [16]. In contrast with the above study, the work by Walsh and colleagues [17] suggested that NOX2 has no relevant role in response to collagen or collagen-related peptide (CRP), with NOX1 playing the main activating role in the signalling of the collagen-specific GPVI receptor. NOX2 only appeared critical for the stabilization of the thrombus [17]. Despite the limitation of relying on a pharmacological inhibitor partially selective for NOX1, the study by Vara and colleagues [15] is in agreement with that of Walsh and colleagues, with NOX1 playing a key activating role in the signalling of GPVI. A recent study by Magwenzi and colleagues [18] described a critical role for NOX2 in the hyperactivity of platelets induced by oxidized low-density lipoprotein (LDL), which is typical of dyslipidemic disorders (e.g. hypercholesterolemia). Oxidised LDL inhibits cGMP-signaling through a mechanism that requires NOX2 and intracellular ROS, which leads to platelet hyperactivity.

Despite the centrality of redox homeostasis in platelet regulation and the growing importance of ROS detection in haemostasis and thrombosis research, there is an alarming paucity of reliable cell assays for the detection of ROS in platelets. Assays with 2',7'-dichlorofluorescein diacetate (DCFDA) are still largely used in the platelet field, despite the relevant limitations of this probe due to the generation of artefacts and its interaction with pharmacological tools often used for cell signalling studies [19, 20]. Dihydroethidium (DHE) has been consistently suggested as a safer alternative to DCFDA [20]. The detection of oxidation products of DHE by liquid chromatography mass spectrometry (LC-MS) is the most reliable approach and it

has been successfully applied to platelets [18]. Unfortunately, the detection of DHE oxidation by LC-MS is extremely laborious and requires equipment and skills usually absent in haemostasis and thrombosis laboratories. Here, we propose and validate an alternative fluorescence probe-based technique for the detection of superoxide anion in platelets based on the intracellular detection of 2-hydroxy-ethidium (2OH-Et⁺), the product of DHE oxidation by superoxide anions. Using this technique, we revealed that the pro-thrombotic activity of amyloid peptide β [21-23] is accompanied by and depends on the pro-oxidative state induced in human platelets via NOX activation.

Methods

Platelet isolation

Human blood was drawn from healthy volunteers by median cubital vein venepuncture under local ethics committee approval. Sodium citrate was used as anti-coagulant (0.5% w/v for platelet isolation - 0.1% w/v for whole blood experiments). Platelet suspensions were obtained as previously described [24]. Briefly, platelet-rich plasma (PRP) was separated from whole blood by centrifugation (200 x g, 15 min), and platelets were separated from PRP by a second centrifugation step (400 x g, 10 min), in the presence of prostaglandin E1 (40 ng/ml) and indomethacin (10 μ M). Platelets were then resuspended in a modified Tyrode's-HEPES buffer (145 mM NaCl, 2.9 mM KCl, 10 mM HEPES, 1 mM MgCl₂, 5 mM glucose, pH 7.3) at a density of 2×10^8 cells/ml.

DHE-based detection of intracellular ROS by flow cytometry

Platelet suspensions obtained as described above were rested for 30 minutes at 37°C before incubation with 5 μ M DHE for 15 minutes. Platelets were then stimulated with the physiological stimuli thrombin (0.01-1.0 unit/ml), CRP (0.1-10 μ g/ml), ADP (1-100 μ M) or arachidonic acid (1-100 μ M) for 15 minutes or with A β peptides (1-100 μ M). Where indicated, a ROS scavenger (i.e. NAC) or an inhibitor (i.e. VAS2870 or indomethacin) was co-incubated with stimuli. At the end of the incubation time, platelet suspensions were diluted 1:10 in cold modified Tyrode's-HEPES buffer. Within 30 minutes from preparation, samples were then analysed using a FACSAria III flow cytometer with 85 μ m nozzle at low flow rate. Gate selection (shown in Figure 3A) was performed using logarithmic side and forward scattering with the gain set at 40 and 220 mV, respectively. Fluorescence excitation was obtained with a violet laser at 405 nm and emission was collected at 580 nm with a LP600 filter. Baseline for unstimulated samples was set at 200 relative fluorescence units. The protocol is summarised schematically in Figure 1B.

DHE-based detection of intracellular ROS by confocal microscopy

Glass coverslips were coated with 0.1 mg/ml fibrillar collagen I from equine tendons (Horm collagen). Platelets were diluted to a density of 4×10^7 /ml and treated with 10 μ M dihydroethidium (DHE). After 1 minute of incubation, platelets were dispensed over the coverslip in a live cell-imaging chamber. Where indicated, ROS scavenger N-acetylcysteine (NAC, 1 mM) or NOX inhibitor VAS2870 (10 μ M) were added to the live cell chamber. Oxidation-dependent probe conversion and fluorescence generation were monitored by confocal imaging for a period of 10 min (405/580 nm ex/em). Images were collected every 10 sec using a Zeiss 510 LSM confocal microscope equipped with a 40x oil immersion lens and single cell fluorescence was quantified using Zeiss LSM Examiner software (Zeiss, Jena, Germany). Single platelet fluorescence values were utilized to construct response curves (mean \pm SEM).

Platelet aggregation assay by suspension turbidimetry

Human platelet suspensions at physiological density (2×10^8 /ml) were stimulated with thrombin (0.01-1 unit/ml), CRP (0.1-10 μ g/ml), ADP (1-100 μ M), arachidonic acid (1-100 μ M) or A β peptides (20 μ M, with scrambled A β peptide 1-42 as a control). The aggregation experiments were performed for 8-10 min, with agonist addition at time 1 min. Where indicated, platelets were preincubated with a ROS scavenger (i.e. 3 mM NAC), an inhibitor (i.e. 10 μ M VAS2870 or 10 μ M indomethacin) or A β peptides (5-20 μ M, with scrambled A β peptide 1-42 as a control). Aggregation was measured by turbidimetry at 37°C using a 490D aggregometer (Chrono-Log Corporation, Havertown, PA), as previously described [15].

Amyloid peptides and fibril formation

Amyloid peptides were synthesised by Life Protein (New Jersey, US). The sequences of the peptides are the following:

A β 1-40: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV (4.3 kDa)

A β 1-42: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA (4.5 kDa)

Scrambled A β 1-42: DEFAKNIGHHDGVAVHMYKGRQVEFIGSIALVFEDVGSAGLV (4.5 kDa)

Peptide fibrillation was obtained by resuspension in phosphate buffer saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3) and incubation for 24 h at 37°C. Thioflavin T was used to monitor fibril formation, as previously described [25].

Results

DHE was utilised as a superoxide anion-specific probe, as previously described [26, 27]. As shown in Figure 1A, DHE is directly oxidised to ethidium by other ROS, while only superoxide anion generates 2OH-Et⁺. Previous studies described the use of liquid chromatography mass spectrometry (LC-MS) to detect 2OH-Et⁺ in platelets [18], which was utilised for the analysis of the redox stress induced by oxidised-LDL in platelets. The use of LC-MS requires time-consuming and expensive analyses and limits the use of DHE-dependent superoxide anion formation analysis to laboratories with access to mass spectrometry facilities. This is a limitation that hinders the application of platelet redox analysis using DHE in several academic and clinical haematology laboratories. Here, we describe the application of spectral properties specific for 2OH-Et⁺ to the analysis of superoxide anion formation by flow cytometry. Only 2OH-Et⁺ (but not ethidium) has an excitation peak around 400 nm wavelength with emission at 580 nm. We took advantage of this property to design a flow cytometry assay specifically aimed at detecting 2OH-Et⁺ and measuring the intracellular rate of superoxide anion generation in platelets (Figure 1B).

As shown in Figure 2, we confirmed the generation of 2OH-Et⁺ as a product of DHE oxidation by superoxide anion in platelets adhering to fibrillar collagen by flow cytometry using the spectral properties specific for this chemical species (i.e. 405/580 nm ex/em). Using single platelet tracking analysis, we could confirm superoxide anion generation in platelets upon adhesion and spreading on fibrillar collagen. The addition of the ROS scavenger NAC (1 mM) or the NOX inhibitor VAS2870 (10 μ M) confirmed that the fluorescence measured in this assay is indeed due to ROS-dependent DHE oxidation and specifically by NOX activity.

We then applied the use of DHE to flow cytometry to assess superoxide anion generation in platelet suspensions (Figure 3). The spectral properties tested by confocal microscopy in Figure 2, were also utilised for the flow cytometry assay (i.e. 405/580 nm ex/em). The gating utilised to analyse platelet is shown in Figure 3A, while an example of 2OH-Et⁺-specific fluorescence before and after thrombin stimulation is shown in Figure 3B. Concentrations of thrombin from 0.1 unit/ml (Figure 3C), CRP from 3 μ g/ml (Figure 3D) and arachidonic acid from 30 μ M (Figure 3E) significantly increased fluorescence levels, while ADP did not significantly increase 2OH-Et⁺ generation (Figure 3F). In order to confirm that the agonists were able to stimulate platelets in the conditions that we chose, platelet aggregation experiments were performed, which confirmed all 4 agonists to be active under our experimental conditions (Figure 3G).

In Figure 4A, the concentration-dependent ablation of thrombin-dependent superoxide anion formation by NAC is shown, with 3 mM completely inhibiting the response. The source of superoxide anion in response to 0.3 units/ml thrombin, 3 μ g/ml CRP and 30 μ M arachidonic was then tested using the pan-NOX inhibitor VAS2870 [28], which at a concentration of 10 μ M is commonly used in vitro to inhibit all members of the NOX family [29, 30]. While 3 mM NAC abolished the response induced by all three agonists, only thrombin and CRP were inhibited by VAS2870, which suggested that these two agonists stimulate superoxide anion generation in a NOX-dependent manner (Figure 4B and C, respectively). In contrast, arachidonic

acid-induced superoxide anion was not affected by VAS2870 (Figure 4D). Interestingly, the cyclooxygenase inhibitor indomethacin inhibited superoxide anion formation in response to arachidonic acid, which reflects the dependence of arachidonic acid responses on conversion of this agonist into prostaglandin H₂ (PGH₂) by cyclooxygenases and the conversion of PGH₂ into thromboxane A₂ by thromboxane synthase [31]. In accordance with these data, thrombin- and CRP-dependent platelet aggregation was inhibited by 10 μ M VAS2870 (Figure 5A-B) and 3mM NAC (data not shown), while arachidonic acid-dependent aggregation was insensitive to VAS2870, but fully inhibited by indomethacin (Figure 5C-D). 3mM NAC only partially inhibited arachidonic acid-dependent aggregation (Figure 5C-D), despite completely scavenging superoxide anion in response to this agonist (Figure 4D).

Finally, we characterised the effect of A β peptides on platelet redox homeostasis. In our hands, only the long version of the peptide (i.e. A β 1-42) had a significant effect on superoxide generation, while 1-40 and the scrambled peptide used as a control had no effect (Figure 6A). A concentration-response study on A β 1-42 peptide, showed that only medium-to-high micromolar concentrations of this peptide induces significant increases in superoxide anion generation (i.e. >10 μ M, Figure 6B). As seen for thrombin and CRP, both ROS scavenger (NAC and NOX inhibitor VAS2870) abolished the A β 1-42 peptide-dependent increase in superoxide anion formation, suggesting that NOXs are required for the stimulation of redox stress in human platelets in response to A β peptide (Figure 6C). We took the opportunity to test the ability of A β 1-42 peptide to induce platelet aggregation. A β 1-42 induced only moderate aggregation at concentration up to 20 μ M (Figure 7A). On the other hand, the effect of A β 1-42 peptide on aggregation when co-applied with submaximal concentrations of another stimulus was a lot more pronounced. Platelet stimulation with 0.03 unit/ml thrombin resulted in negligible aggregation, but in the presence of 5 and especially 20 μ M of A β 1-42 peptide resulted in a strong aggregation response (Figure 7B). Similarly, 10 μ M arachidonic acid only induced around 30% aggregation, but the aggregation increased to almost 80% in the presence of 20 μ M A β 1-42 peptide (Figure 7C-D). Interestingly, in the presence of 10 μ M VAS2870 A β 1-42 peptide did not potentiate arachidonic acid-dependent platelet aggregation, suggesting that NOX activity is necessary for the effect of A β peptide on human platelets. Unfortunately, similar experiments were not possible with thrombin or CRP as platelet activation in response to these two stimuli depends on NOX activity and in the presence of VAS2870 platelet activation is completely abolished.

Discussion

The detection of ROS and specifically superoxide anion in platelets is important to understand the redox dependence of haemostatic response and its dysregulation in thrombotic diseases [32]. Nonetheless, this field of research is suffering from the lack of reliable and standardized methodologies for the measurement of intracellular ROS in platelets. This makes impossible the comparison of data between studies and ultimately slows down the development of this biomedical research area. DCFDA is

still widely used in the platelet field despite its relevant limitations due to the generation of artefacts and the interaction with pharmacological tools often used for cell signalling studies [19, 20]. More reliable methodologies are available but require specific skills or equipment that are not readily available in academic and clinical laboratories. Amongst these alternatives, electron paramagnetic resonance (EPR) spectroscopy is considered the gold standard for the detection of intracellular ROS [33]. Unfortunately, in addition to extremely low throughput due to procedural complexities and lengthy data analysis, this technique requires highly specialised equipment and dedicated personnel, which limit its widespread adoption in research and clinical practice. For these reasons the use of redox-sensitive fluoroprobes remains attractive, especially if combined with commonly used and relatively accessible detection techniques such as cell imaging or flow cytometry. Amongst available fluoroprobes, DHE has been described as the most reliable tool for ROS detection in live cells [20]. The detection of oxidation products of DHE by liquid chromatography mass spectrometry (LC-MS) has been described in several studies, including some platelet studies [18], but like EPR spectroscopy it is not readily available in most academic and clinical laboratories. Here we describe a methodology that combines the reliability of DHE as a redox-sensitive fluoroprobe with the advantages of flow cytometry as a detection method (i.e. user-friendliness, high throughput and wide adoption in academic research and clinical practice). We demonstrated the reliability and usefulness of this technique by performing a detailed pharmacological study on the redox properties of physiological platelet agonists (including collagen, thrombin, ADP and arachidonic acid) and the pro-thrombotic activity of A β peptides.

In this study we confirmed that platelet activation is associated with ROS generation. In agreement with previous studies, thrombin [11, 34, 35], collagen (and CRP) [15, 17] and arachidonic acid [36] were shown to induce superoxide anion generation. From these data, it seemed that platelet aggregation and superoxide anion generation were intimately associated, but this hypothesis was opposed by our data with ADP, which appeared to stimulate platelet aggregation but had no effect on platelet superoxide anions. This was somewhat corroborated by literature, in which a link between ADP-dependent platelet activation and ROS generation has not been convincingly shown. Thanks to the use of a specific NOX inhibitor we could prove that NOXs are critical for the oxidative stress induced in platelets by thrombin and collagen, which was previously shown [11, 15, 17, 37]. For the first time, we demonstrated that the superoxide anion formation induced by arachidonic acid is NOX-independent. Aggregation experiments confirmed that arachidonic acid-induced signalling (which is mediated by conversion of this molecule into thromboxane A₂ [31]) as well as ADP-mediated platelet activation are independent of NOX activity and ROS generation. In these experiments, the ROS scavenger NAC only partially inhibited arachidonic acid-dependent aggregation and no effect on ADP-dependent aggregation, while NOX inhibitor VAS2870 had no effects on either arachidonic- or ADP-dependent platelet aggregation. This suggests the possibility of obtaining agonist-specific platelet inhibition of platelets by NOX inhibitors. This was

proposed previously [15, 17], although the differential role of NOX1 and NOX2 in response to different agonists remains controversial [15, 16, 38].

Finally, we utilised the flow cytometry-based detection of superoxide anion formation by DHE to clarify the molecular mechanism underlying the pro-thrombotic activity of A β peptides [21]. We demonstrated a significant association between platelet exposure to A β peptides and oxidative stress, which has been described [39]. Importantly, in this study we identified superoxide anions as the ROS induced by A β peptides and, more importantly, we show that NOXs are crucial for oxidative stress and platelet aggregation potentiation induced by A β peptides. This is an interesting finding suggesting NOX as potential therapeutic targets to address the pro-thrombotic activity of A β peptide [40] and treat neurovascular ischemia associated with Alzheimer's disease [41, 42]. Although recent studies focussed on different A β peptides for their effects on platelet function (e.g. A β 25-35 [22], A β 1-40 [21]), only A β peptide 1-42 appeared to significantly stimulate superoxide anion formation. This is an extremely interesting observation as it may suggest specific binding modalities for the more hydrophobic A β peptide 1-42 compared to A β peptide 1-40.

In summary, here we describe a novel, reliable and easily accessible methodology for the measurement of superoxide anion formation in platelets. Its application in our study led to interesting observations on the redox properties of physiological platelet agonists and A β peptides, which highlights how useful this assay can be in the study of haemostasis and thrombosis.

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Declaration of Interest statement

The authors declare no conflict of interest.

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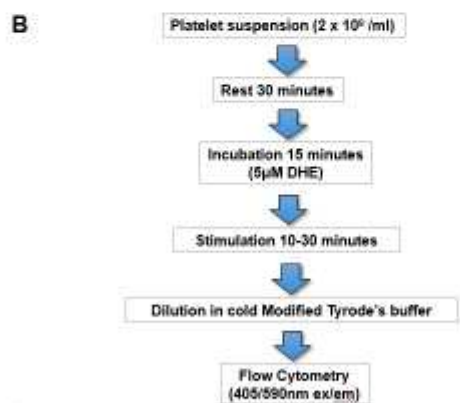
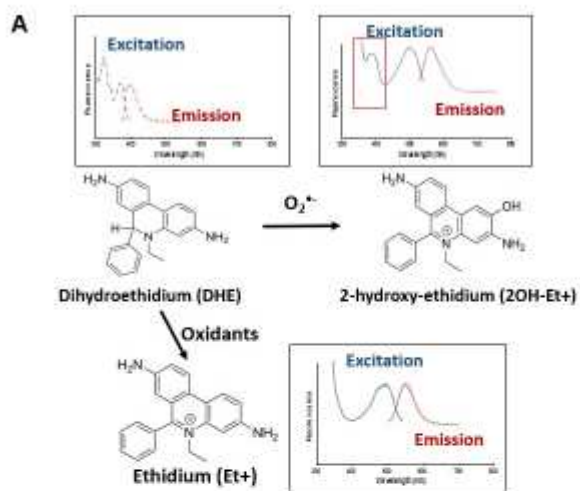


Figure 1

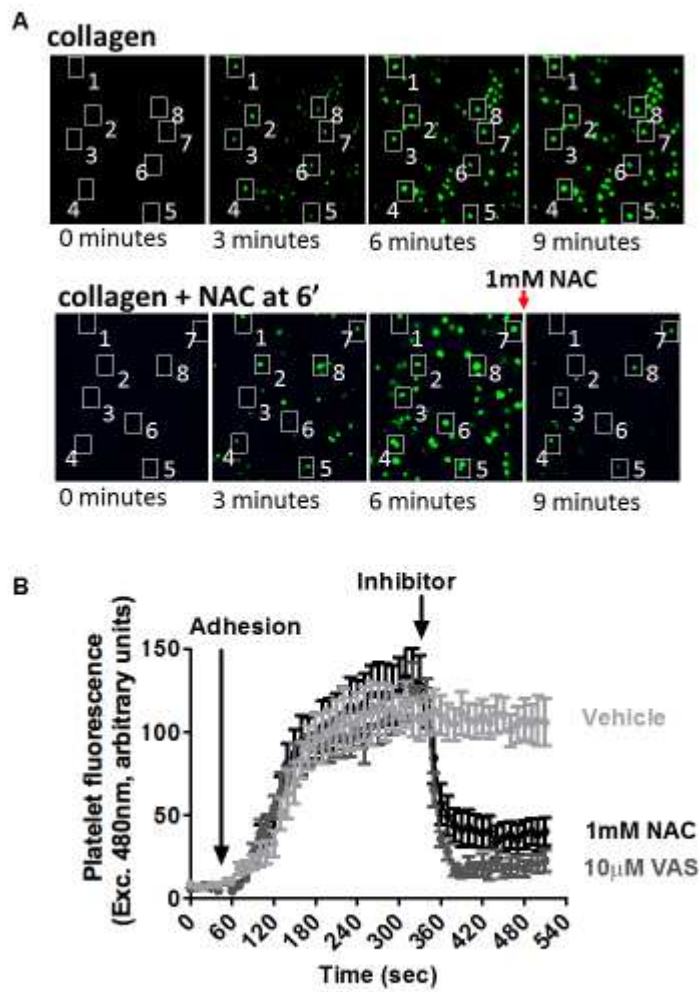


Figure 2

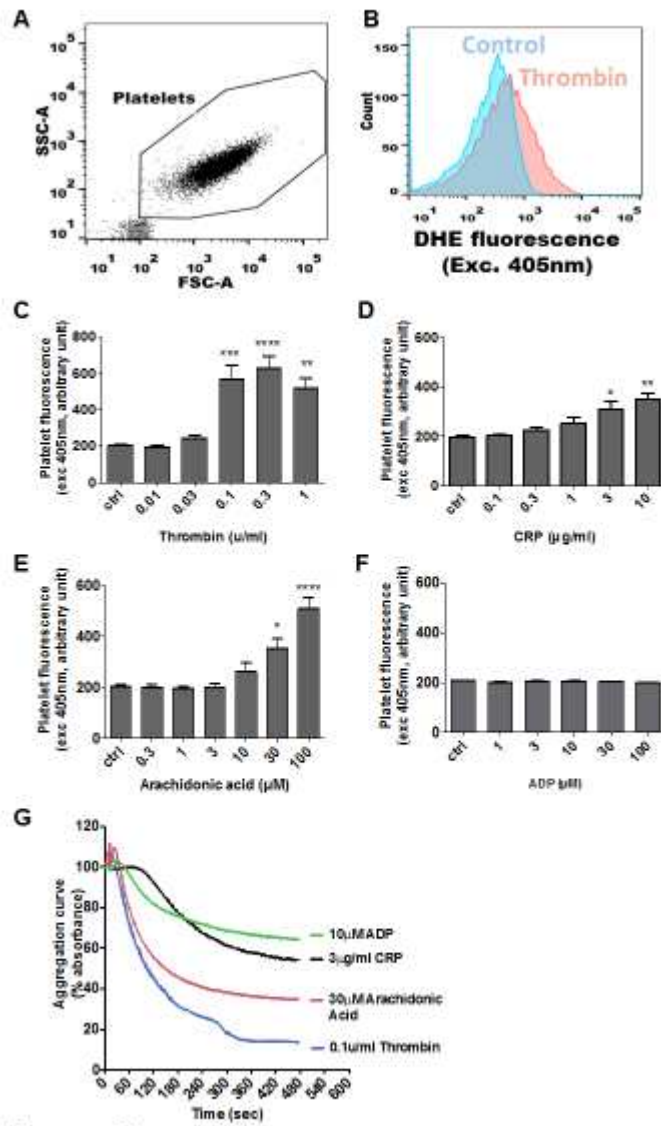


Figure 3

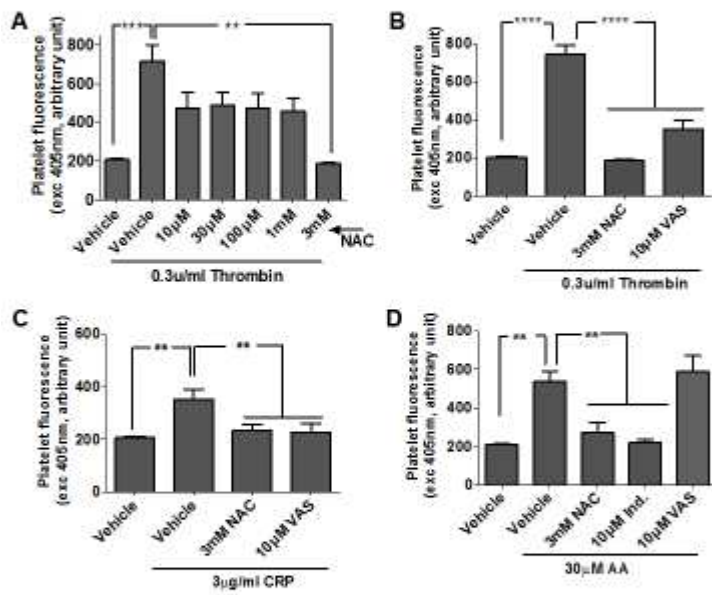


Figure 4

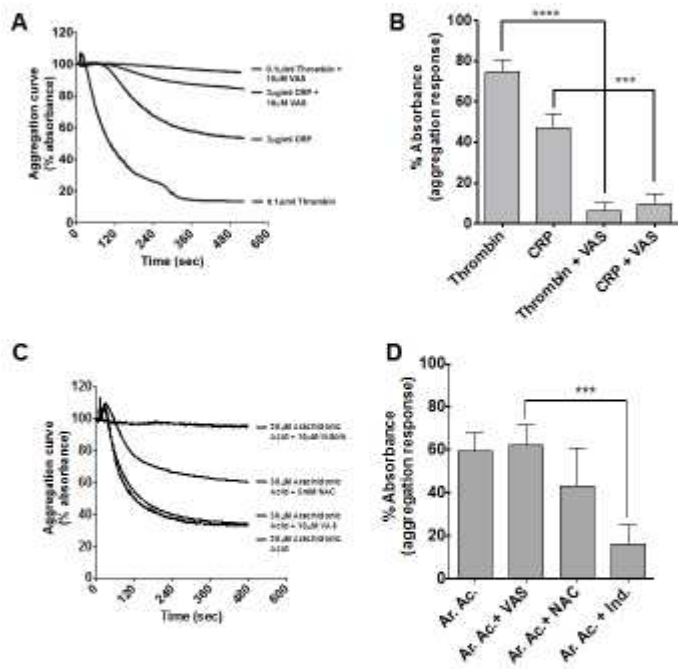


Figure 5

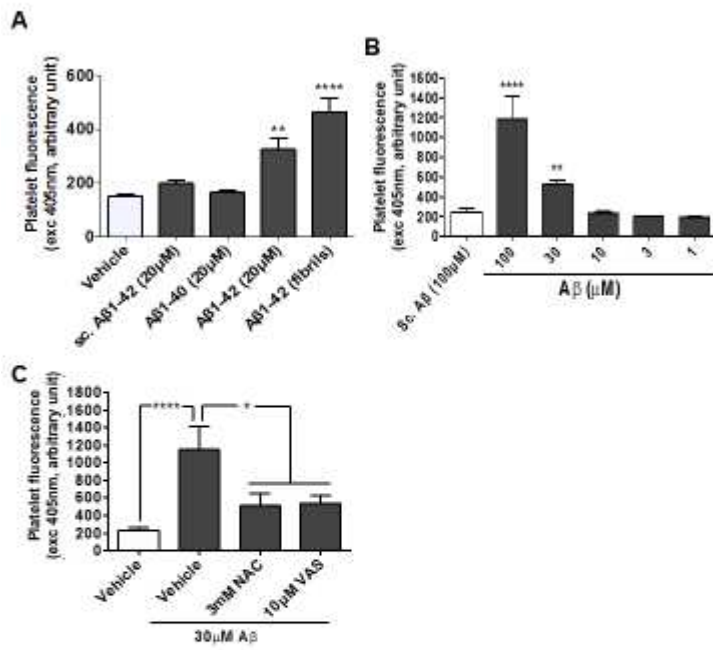


Figure 6

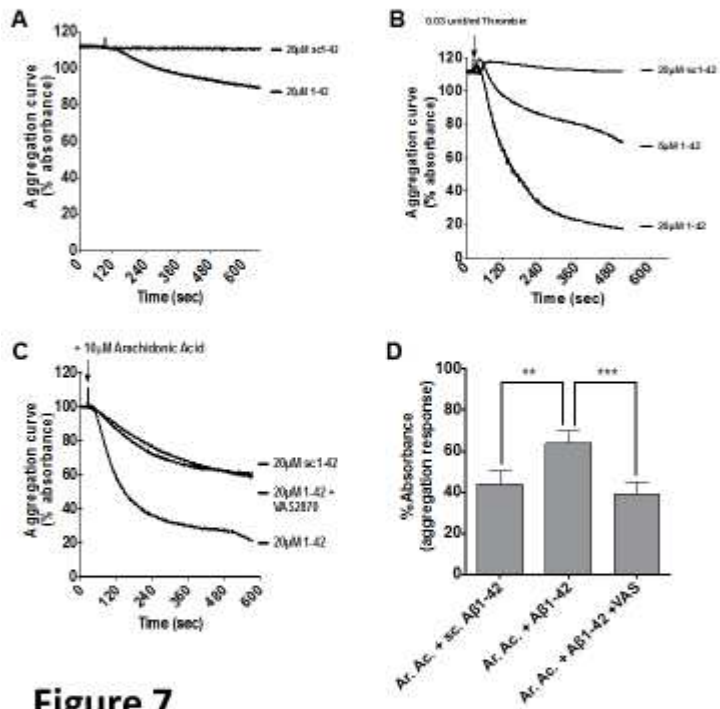


Figure 7