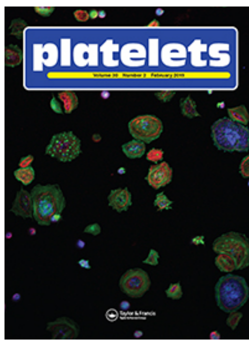

Platelets

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ORIGINAL ARTICLE

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

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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 hemostasis and thrombosis. Nonetheless, despite several assays have been described and successfully utilized in the past, the analysis of ROS generation in human platelets remains challenging.

Here we show that dihydroethidium (DHE) allows the characterization 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. 0.1unit/ml thrombin, 3 µg/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.

In addition, the pathological platelet stimulus amyloid β (Aβ) 1–42 peptide induced superoxide anion formation in a concentration-dependent manner. Aβ peptide stimulated superoxide anion formation in a NOX-dependent manner, as proved by the use of VAS2870. Aβ 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 Aβ-dependent potentiation of platelet aggregation in response to 10 µM arachidonic acid, suggesting that the pro-thrombotic activity of Aβ peptides depends on NOX activity. Similar experiments could not be performed with thrombin or collagen, as NOXs are required for the signaling induced by these stimuli. These findings shed some new light on the pro-thrombotic activity of Aβ 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.

Keywords

Amyloid peptide β, NADPH, NOX, Platelet, redox, superoxide anion

History

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Introduction

The generation of superoxide anion by platelets was described over 40 years ago [1]. In recent years, reactive oxygen species (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 hemostasis modulation [8,9].

Several molecular mechanisms have been described that underlie 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

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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 signaling [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 NOX2 [15]. ROS generated by NOX1 and NOX2 promote platelet activation via the Syk/phospholipase Cγ2/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 signaling 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 signaling 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). Oxidized 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 hemostasis 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 artifacts and its interaction with pharmacological tools often used for cell signaling 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 hemostasis 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 µM). 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 µM DHE for 15 minutes. Platelets were then stimulated with the physiological stimuli thrombin (0.01–1.0 unit/ml), CRP (0.1–10 µg/ml), ADP (1–100 µM) or arachidonic acid (1–100 µM) for 15 minutes or with Aβ peptides (1–100 µM). 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 analyzed using a FACSAria III flow cytometer with 85 µm 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 summarized 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 x 10^7/ml and treated with 10 µM 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 µM) 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 seconds 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 ± SEM).

Platelet aggregation assay by suspension turbidimetry

Human platelet suspensions at physiological density (2 x 10^8/ml) were stimulated with thrombin (0.01–1 unit/ml), CRP (0.1–10 µg/ml), ADP (1–100 µM), arachidonic acid (1–100 µM) or Aβ peptides (20 µM, with scrambled Aβ 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 µM VAS2870 or 10 µM indomethacin) or Aβ peptides (5–20 µM, with scrambled Aβ 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 synthesized by Life Protein (New Jersey, US). The sequences of the peptides are the following:

Aβ1–40:DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAII GLMVGGVV (4.3 kDa)

Aβ1–42: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAII GLMVGGVVIA (4.5 kDa)

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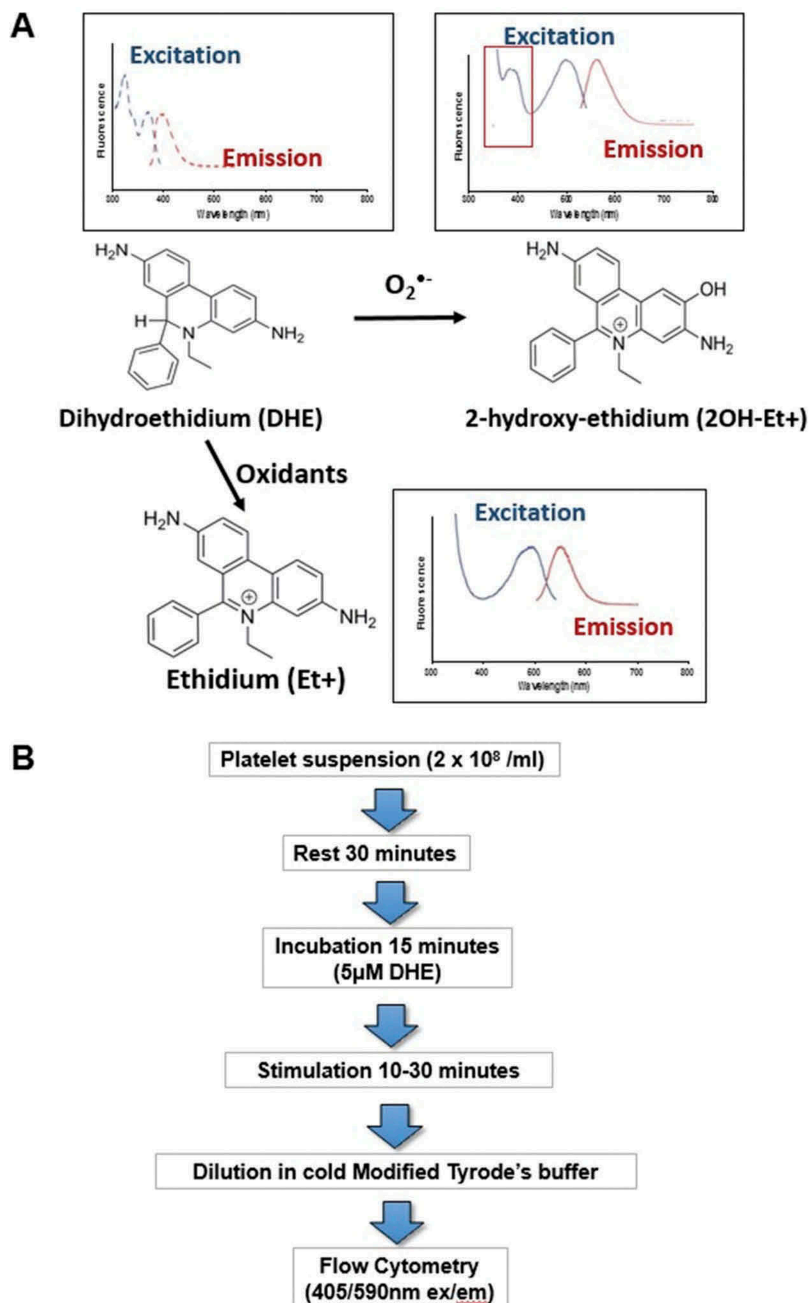

Figure 1. (A) Structure and spectral properties of DHE and its two oxidation products ethidium and 2-hydroxy-ethidium (2OH-Et+). The excitation peak at 405 nm specific for 2OH-Et+ is highlighted by a red rectangle. (B) Schematic representation of the DHE-based flow cytometric detection of intracellular superoxide anions in platelets.

Scrambled Aβ1–42: DEFAKNIGHHDGVAVHMYKGRQVE FIGSIALVFEDVGSAGLV (4.5 kDa)

Peptide fibrillation was obtained by resuspension in phosphate buffer saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 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 utilized as a superoxide anion-specific probe, as previously described [26,27]. As shown in Figure 1A, DHE is directly oxidized 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 utilized for the analysis of the redox stress induced by oxidized-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 hematology 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

Platelets, 2019; 30(2): 181–189

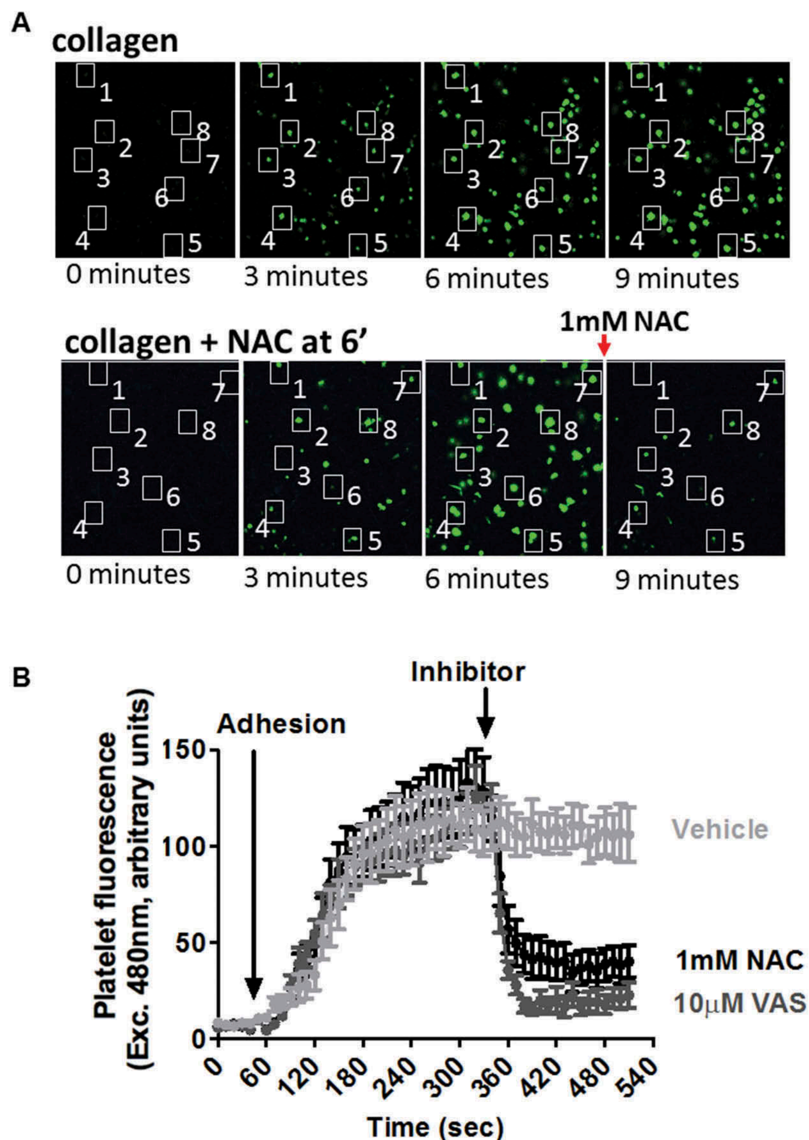

Figure 2. Utilization of DHE to detect superoxide anion formation in platelets adhering to collagen by confocal microscopy. Platelet suspensions were treated for 1 minute with 10 µM dihydroethidium (DHE), then platelets were dispensed over the coverslip in a live cell-imaging chamber. Where indicated, ROS scavenger 1 mM N-acetylcysteine (NAC) or NOX inhibitor 10 µM VAS2870 were added to the live cell chamber after 6 minutes. Representative images at times 0 min, 3 min, 6 min and 9 min are shown in A, while fluorescence quantification by single platelet tracking is shown in B. Aggregation results are representative of four independent experiments.

fibrillar collagen. The addition of the ROS scavenger NAC (1 mM) or the NOX inhibitor VAS2870 (10 µM) 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 utilized for the flow cytometry assay (i.e. 405/580 nm ex/em). The gating utilized to analyze 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 0.1unit/ml (Figure 3C), CRP from 3µg/ml (Figure 3D) and arachidonic acid from 30 µM (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 µg/ml CRP and 30 µM arachidonic was then tested using the pan-NOX inhibitor VAS2870 [28], which at a concentration of 10 µM 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 µM VAS2870

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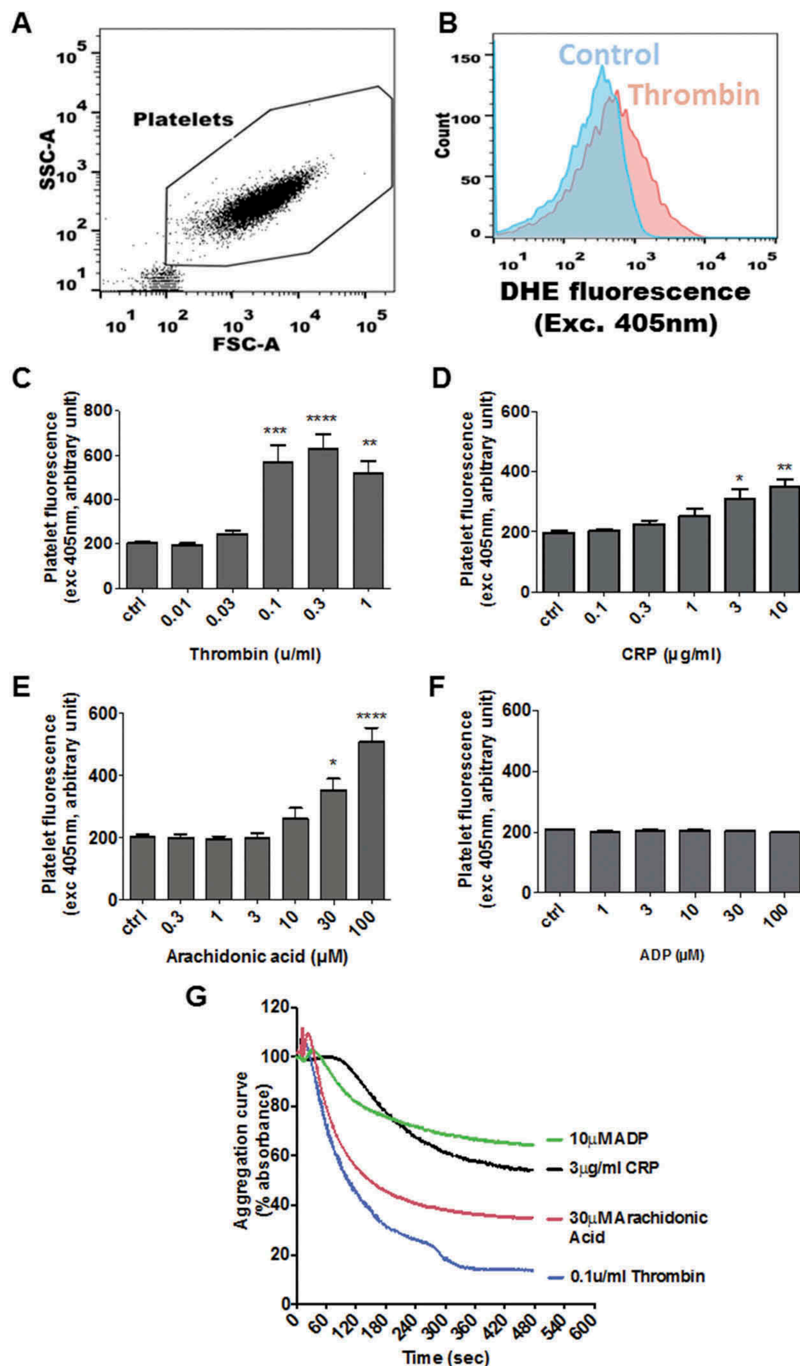


Figure 3. Utilization of DHE to detect superoxide anion formation in platelets stimulated in suspension by thrombin, CRP, arachidonic acid or ADP. Flow cytometry gating is shown in a forward (FSC) vs side scattering (SSC) plot in A. A representative example of the fluorescence shift by thrombin-dependent stimulation of platelets in the 2OH-Et+ fluorescence (405/580 nm ex/em) is shown in B. Platelet suspensions obtained as described above were rested for 30 minutes at 37°C before incubation with 5 μM DHE for 15 minutes. Platelets were then stimulated with physiological stimuli: thrombin (0.01–1.0 unit/ml) (C), CRP (0.1–10 μg/ml) (D), ADP (1–100 μM) (E) or arachidonic acid (1–100 μM) (F) for 15 minutes. Means ± standard error of the means (SEM) of platelet fluorescence is shown (from 3 or more independent experiments). Statistical significance for 4 independent experiments was analyzed by one-way ANOVA with Bonferroni post-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). Platelet aggregation induced by indicated conditions was monitored for up to 10 minutes by turbidimetry (G). These results are representative of four independent experiments.

(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 characterized the effect of Aβ peptides on platelet redox homeostasis. In our hands, only the long version of the peptide (i.e. Aβ1–42) 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 Aβ 1-42 peptide, showed that only medium-to-high micromolar concentrations of this peptide induces significant increases in superoxide anion generation (i.e. >10 μM, Figure 6B). As seen for thrombin and CRP, both ROS scavenger (NAC and NOX inhibitor VAS2870 abolished the Aβ1-42 peptide-dependent increase in superoxide anion formation, suggesting that NOXs are required for the stimulation of redox stress in human platelets in response to Aβ peptide (Figure 6C). We took the opportunity to test the ability of Aβ1-42 peptide to induce platelet aggregation. Aβ1-42 induced only moderate aggregation at concentration up to 20 μM (Figure 7A). On the other hand, the effect of Aβ1-42 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 μM of Aβ1-42 peptide resulted in a strong aggregation response (Figure 7B). Similarly, 10 μM arachidonic acid only induced around 30% aggregation, but the aggregation increased to almost 80% in the presence of 20 μM Aβ1-42 peptide (Figure 7C-D). Interestingly, in the presence of 10 μM VAS2870 Aβ1-42 peptide did not potentiate arachidonic acid-dependent platelet aggregation, suggesting that NOX activity is necessary for the effect of Aβ peptide on human platelets.

Platelets, 2019; 30(2): 181–189

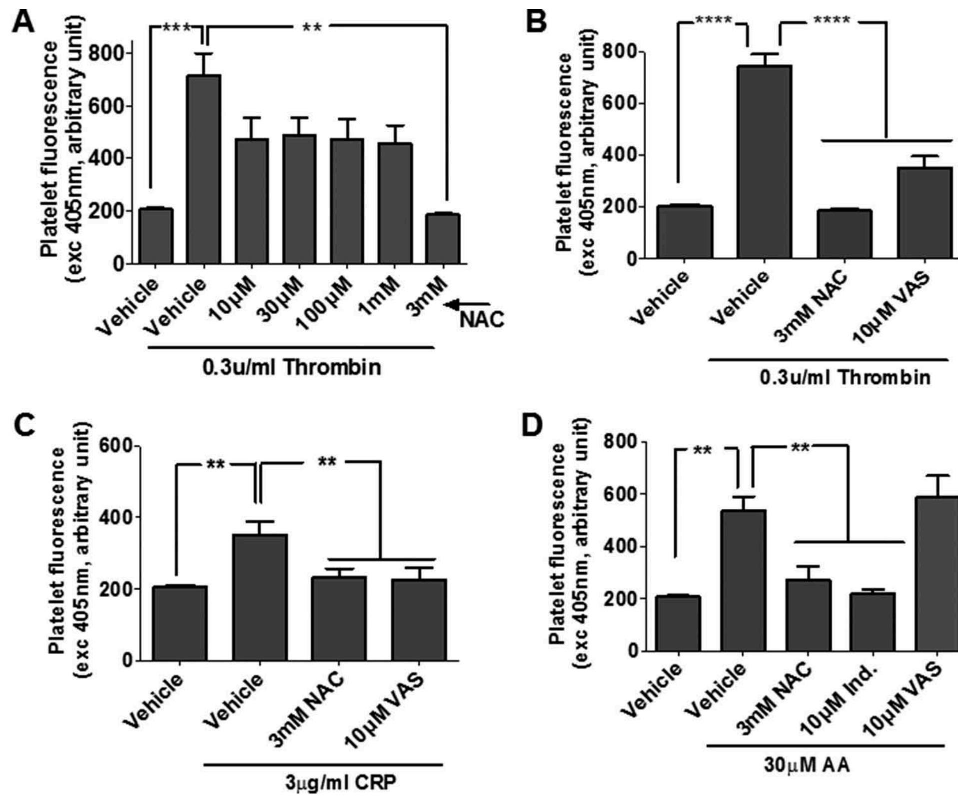
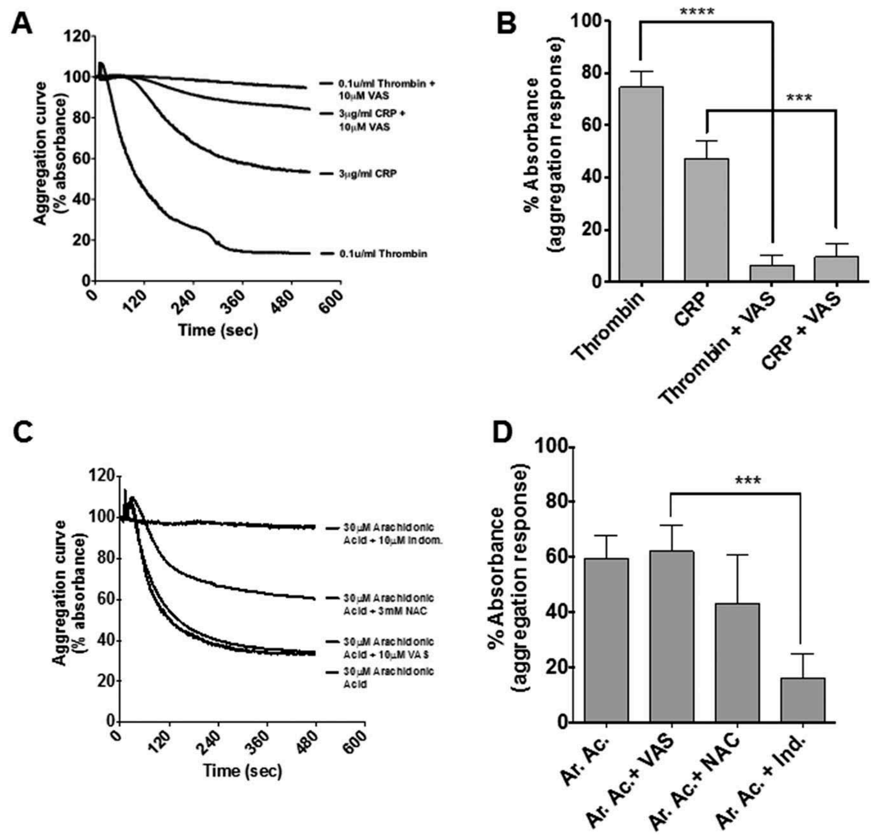


Figure 4. NOX-dependence of the superoxide anion formation induced by physiological platelet agonists. Superoxide anion formation was measured as described above in response to 0.3 units/ml thrombin (A-B), 3 μg/ml CRP (C) or 30 μM arachidonic acid (D) following 10 min pre-incubation with NAC (10 μM-3 mM) or pan-NOX inhibitor VAS2870 (10 μM). Statistical significance for 4 independent experiments was analyzed by one-way ANOVA with Bonferroni post-test (* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001).

Figure 5. NOX-dependence of platelet aggregation in response to CRP and thrombin. Platelet aggregation induced by 0.1 units/ml thrombin and 3 μg/ml CRP (A) or 30 μM arachidonic acid (C) was monitored for up to 10 minutes by turbidimetry in the presence of 3 mM NAC, 10 μM VAS2870 or 10 μM indomethacin, as indicated. Aggregation results from A and C were quantified in B and D, respectively. Statistical significance for 4 independent experiments was analyzed by one-way ANOVA with Bonferroni post-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).



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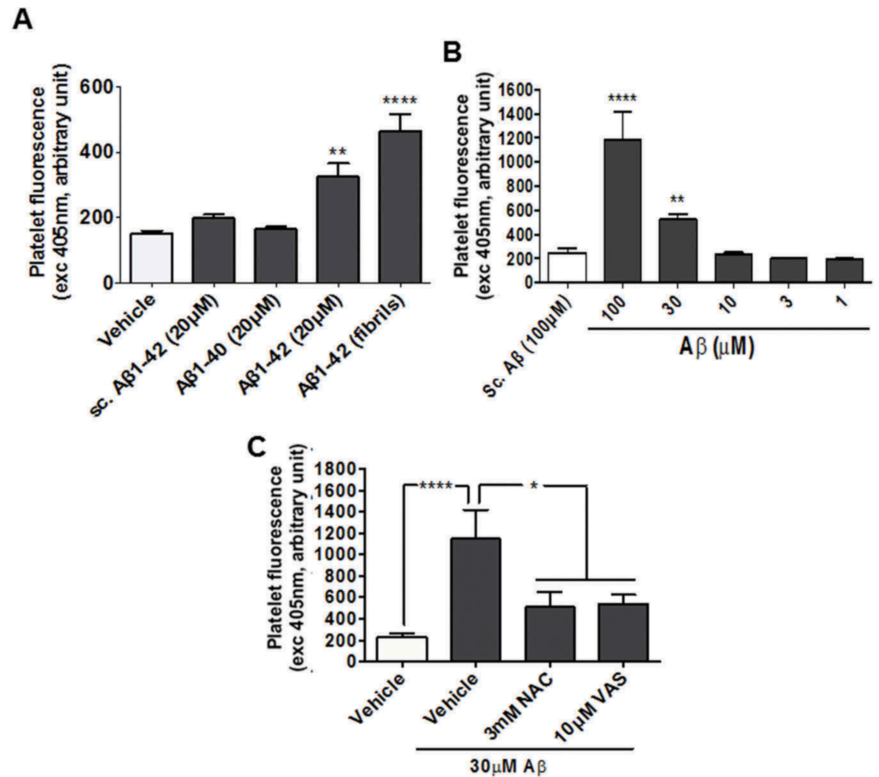

Figure 6. Superoxide anion formation induced by Aβ peptides. Superoxide anion formation was measured as described above in response to 20 μM Aβ1–40, Aβ1–42, scrambled Aβ1–42 or a preparation of Aβ1–42 fibrils (A). Different concentrations of Aβ1–42 (1–100 μM) were also tested (B). The superoxide anion formation stimulated by 20 μM Aβ1–42 is inhibited by 10 min pre-incubation with NAC (10 μM-3 μM) or 10 μM VAS2870. Statistical significance for 4 independent experiments was analyzed by one-way ANOVA with Bonferroni post-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

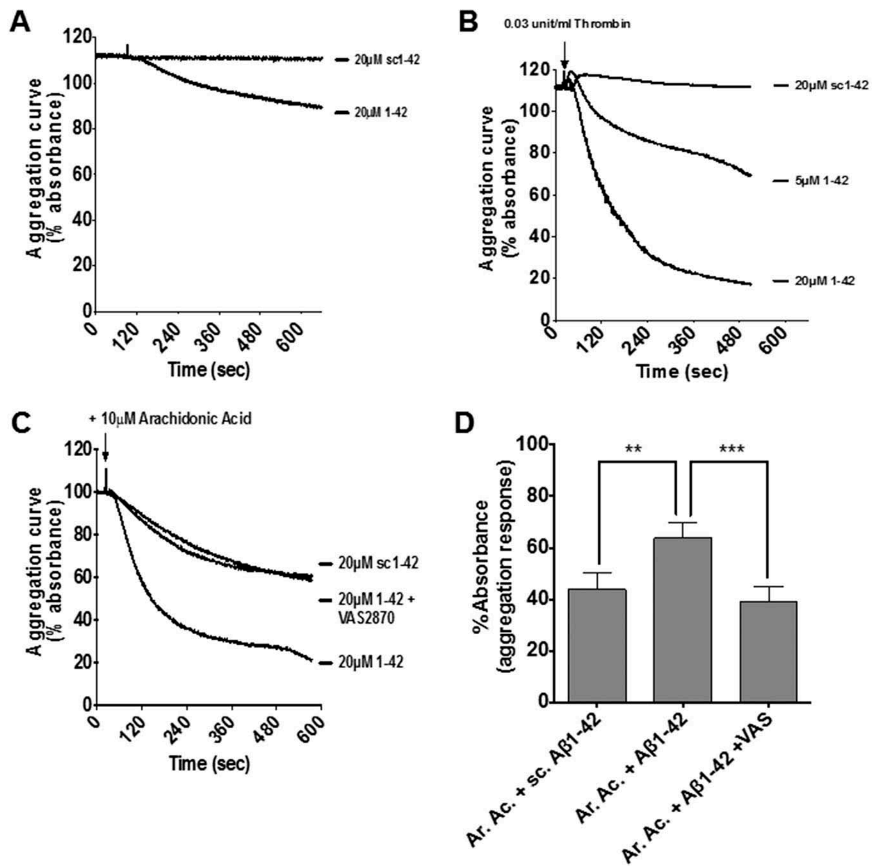

Figure 7. Potentiation of platelet aggregation by Aβ1–42 and its NOX-dependence. Platelet aggregation induced by 20 μM Aβ1–42 or scrambled Aβ1–42 is shown in (A). The effect of pre-incubation with 5 or 20 μM Aβ1–42 or scrambled Aβ1–42 on aggregation stimulated by 0.03 units/ml thrombin is shown in B. The effect of pre-incubation with 20 μM Aβ1–42 or scrambled Aβ1–42 or 20 μM Aβ1–42 plus 10 μM VAS2870 on aggregation stimulated by 10 μM arachidonic acid is shown in C. Aggregation results from C were quantified in D. Statistical significance for 4 independent experiments was analyzed by one-way ANOVA with Bonferroni post-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

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 hemostatic 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 artifacts and the interaction with pharmacological tools often used for cell signaling 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 specialized 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 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 Aβ 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 signaling (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 utilized the flow cytometry-based detection of superoxide anion formation by DHE to clarify the molecular mechanism underlying the pro-thrombotic activity of Aβ peptides [21]. We demonstrated a significant association between platelet exposure to Aβ peptides and oxidative stress, which has been described [39]. Importantly, in this study we identified superoxide anions as the ROS induced by Aβ peptides and, more importantly, we show that NOXs are crucial for oxidative stress and platelet aggregation potentiation induced by Aβ peptides. This is an interesting finding suggesting NOX as potential therapeutic targets to address the pro-thrombotic activity of Aβ peptide [40] and treat neurovascular ischemia associated with Alzheimer's disease [41,42]. Although recent studies focussed on different Aβ peptides for their effects on platelet function (e.g. Aβ 25–35 [22], Aβ 1–40 [21]), only Aβ 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 Aβ peptide 1-42 compared to Aβ 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 Aβ peptides, which highlights how useful this assay can be in the study of hemostasis and thrombosis.

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Declaration of interest

The authors declare no conflict of interest.

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