Brief report
Platelet NAD(P)H-oxidase–generated ROS production regulates αIIbβ3-integrin activation independent of the NO/cGMP pathway
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Platelets play a crucial role in the physiology of primary hemostasis and pathophysiologic processes such as arterial thrombosis. Accumulating evidence suggests a role of reactive oxygen species (ROSs) in platelet activation. Here we show that platelets activated with different agonists produced intracellular ROSs, which were reduced by reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidase inhibitors and superoxide scavengers. In addition, we demonstrate that ROSs produced in platelets significantly affected αIIbβ3 integrin activation but not alpha and dense granule secretion and platelet shape change. Thrombin-induced integrin αIIbβ3 activation was significantly decreased after pretreatment of platelets with NAD(P)H oxidase inhibitors (diphenylene iodonium [DPI] [45% ± 9%] and apocynin [43% ± 11%]) and superoxide scavengers (tiron [60% ± 9%] and Mn(III)tetrakis (1-methyl-4-pyridyl)porphyrin [MnTMPyP] [70% ± 6%]). These inhibitors also reduced platelet aggregation and thrombus formation on collagen under high shear and achieved their effects independent of the nitric oxide/cyclic guanosine monophosphate (NO/cGMP) pathway.

Study design

Measurement of ROS production
Platelets and neutrophils were isolated from whole blood obtained from healthy volunteers as described previously and informed consent was provided according to the Declaration of Helsinki. For detection of intracellular ROSs, platelets and neutrophils were preloaded with 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) (Molecular Probes, Göttingen, Germany) and measured by flow cytometry (FACSCalibur; Becton Dickinson, Heidelberg, Germany). Extracellular ROS production was measured by L-012 (100 μM; Wako, Neuss, Germany) in chemiluminescence assay (Wallac Victor 1420, Perkin Elmer Wallac Life and Analytical Sciences, Boston, MA).

Analysis of αIIbβ3 activation, P-selectin expression, aggregation, and flow chamber experiments
For the determination of P-selectin expression, washed platelets were stained with R-phycocerythrin (RPE)-conjugated anti-CD62P antibody (BD Biosciences, Heidelberg, Germany). Platelet aggregation and P-selectin expression were measured by the ADP-induced platelet aggregation test (PAP; Haemonetics, Woburn, MA) and the flow chamber (FM800; Haemonetics) at shear rates of 1000 and 1500 s⁻¹. Thrombus formation was assessed using the platelet thrombus formation test (PTFT; Specac, Eching, Germany). Platelet thrombus formation was expressed as the mean thrombus area.

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Supported in part by the Deutsche Forschungsgemeinschaft and by a grant from the Bayerische Forschungsstiftung.

A.J.B. was responsible for platelet experiments, analysis, and writing initial drafts of the paper (as part of her PhD thesis work); S.G. was PhD advisor for A.J.B., analyzed data, and helped write the paper; J.G. advised in the design and interpretation of platelet reactive oxygen species (ROS) experiments; B.A. and M.P. provided advice, help, and analysis of the blood-flow experiments; B.N. provided advice on the blood-flow experiments and helped write parts of the paper; and U.W. was responsible for the initial plan of the work, advising A.J.B. in her PhD work, analyzing data, and writing and finalizing the paper.

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(DAKO, Glostrup, Denmark) and, for αIIBβ3 activation, with fluorescein isothiocyanate (FITC)–PAC-1 antibody (Becton Dickinson). Aggregation was carried out using a Bio/Data PAP-4 aggregometer (Bio/Data, Horsham, PA) with platelet-rich plasma as previously described.10

Flow chamber experiments were carried out as described previously,12 with coverslips coated with Horn-type collagen under high shear conditions (1000 s⁻¹, 4 minutes) with the anticoagulated whole blood.

Serotonin secretion, thromboxane synthase activity, and cGMP and cAMP levels

Analysis of serotonin secretion and thromboxane synthase activity was determined as described previously.13 Levels of cGMP and cyclic adenosine monophosphate (cAMP) were measured in washed platelets according to the manufacturer's protocol (R&D Systems, Wiesbaden-Nordenstadt, Germany).

Data analysis

All experiments were performed at least in triplicate, and data shown are means ± SD. Data were analyzed using analysis of variance (ANOVA) followed by the Bonferroni test or Student t test. Differences were considered significant when the significance was P < .05.

Results and discussion

In previous studies, the production of ROSs was mainly observed with collagen-stimulated5-14 and thrombin-stimulated7-11 platelets. Here we show intracellular ROS production also with thrombin receptor activating peptide 6 (Trap6) and the stable thromboxane A2 analog U46619, but not with adenosine diphosphate (ADP) stimulation (Figure 1A), while no extracellular signal was observed (not shown). Control experiments were done in neutrophils, which produced both intracellular and extracellular ROSs after phorbol myristate acetate (PMA) stimulation (Figure 1A).

To investigate the possible source and type of intracellular ROS production, we used different inhibitors and superoxide scavengers. Inhibitors of mitochondrial respiration, xanthine oxidase, and nitric oxide synthase (NOS) had no effect on ROS production, while NAD(P)H oxidase inhibitors, diphenylene iodonium (DPI) and apocynin, and the cyclooxygenase (COX) inhibitor acetalsalicylic acid (ASA) significantly inhibited thrombin-induced ROS production (Figure 1B), indicating that NAD(P)H oxidase and COX are the likely sources of ROSs in platelets. However, COX is a part of prostaglandin endoperoxide H synthase (PGHS), which produces peroxo compounds like 12(R)-hydroxyperoxy-eicosatetraenoic acid (15(R)-HPETE) and could in turn oxidize H₂DCF-DA. Furthermore, non-ROS–related enzymatic oxidation of H₂DCF-DA could occur, as shown by Larsen et al.17 The lack of effect of NAD(P)H oxidase inhibitors and superoxide scavengers on thromboxane synthase activity (not shown) and the additive inhibition of ROSs by DPI and ASA or tiron and ASA (not shown) indicate that NAD(P)H oxidase–generated ROSs are independent from COX.

That the intracellular superoxide scavengers tiron and superoxide dismutase (SOD) mimetic Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP), but not the hydroxyl scavenger mannitol, inhibited ROS production also indicates superoxide as the major radical in platelets.

To address the role of ROSs in platelet regulation, we assessed the major markers of platelet activation, namely P-selectin expression and dense granule secretion (serotonin), shape change, platelet aggregation, thrombus formation, and integrin activation (Figure 2). Platelet aggregation induced by Trap6 was significantly reduced in the presence of DPI and apocynin (Figure 2A). Aggregation traces showed no loss of shape change even when high concentrations of DPI (300 μM) and apocynin (4.8 mM) were used (not shown). This was further confirmed by unaltered myosin light chain (MLC) phosphorylation and Rho family small G protein RhoA activation (not shown). Integrin αIIBβ3 activation plays a major role in the regulation of platelet adhesion and aggregation. Integrin αIIBβ3 activation was inhibited by NAD(P)H oxidase inhibitors and superoxide scavengers. ASA, as previously shown,18,19 had no effect on integrin activation, indicating that peroxo compounds do not play a significant role in integrin activation. At high shear, activated integrins are a key step in platelet-platelet interactions and thrombus formation. Flow chamber experiments over a collagen-coated surface showed significantly reduced thrombus formation under high shear flow conditions (1000 s⁻¹) in the presence of DPI or apocynin (Figure 2D). Furthermore, platelet stimulation by thrombin, Trap6, U46619, convulxin, and ADP showed a similar pattern of αIIBβ3 activation and ROS production (Figure 2C). Integrin inhibition by ROSs was independent of Rap1 (small guanosine triphosphatase [GTPase] activated by many different platelets agonists) stimulation because no changes in

![Figure 1](image-url)
Rap1b activation were observed (not shown). NAD(P)H oxidase inhibitors and superoxide scavengers had no significant effect on alpha and dense granule secretion (not shown).

Several studies suggested that intracellularly produced ROSs scavenge endothelial or platelet-derived NO in a fast reaction forming peroxynitrite (ONOO\(^{-}\)) as an end product.\(^{7,9,20}\) The NO/cGMP pathway is a well-established mechanism of platelet inhibition and can be monitored by cGMP-dependent protein kinase (cGK)– and cAMP-dependent protein kinase (cAK)–mediated VASP phosphorylation.\(^{21}\) Therefore, we hypothesized that inhibition of ROS production in platelets might lead to an increase of cGMP levels and VASP phosphorylation, because more NO should be present. VASP phosphorylation closely correlates with the inhibition of the integrin IIb3 activation on human platelets.\(^{22}\) However, changes in VASP phosphorylation or GMP levels (Figure 2B) or cAMP (not shown) levels were not observed. Collectively, our data suggest that NAD(P)H oxidase–generated ROSs are involved in integrin activation and act by other mechanisms than scavenging NO. Although the molecular mechanism of integrin activation following cellular activation and ROS production is presently not clear, our results strongly suggest an involvement of intracellular ROSs in integrin regulation.

References


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