



Regulation of platelet activation and thrombus formation by reactive oxygen species



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ABSTRACT

Reactive oxygen species (ROS) are generated within activated platelets and play an important role in regulating platelet responses to collagen and collagen-mediated thrombus formation. As a major collagen receptor, platelet-specific glycoprotein (GP)VI is a member of the immunoglobulin (Ig) superfamily, with two extracellular Ig domains, a mucin domain, a transmembrane domain and a cytoplasmic tail. GPVI forms a functional complex with the Fc receptor γ -chain (FcR γ) that, following receptor dimerization, signals via an intracellular immunoreceptor tyrosine-based activation motif (ITAM), leading to rapid activation of Src family kinase signaling pathways. Our previous studies demonstrated that an unpaired thiol in the cytoplasmic tail of GPVI undergoes rapid oxidation to form GPVI homodimers in response to ligand binding, indicating an oxidative sub-membranous environment in platelets after GPVI stimulation. Using a redox-sensitive fluorescent dye (H₂DCF-DA) in a flow cytometric assay to measure changes in intracellular ROS, we showed generation of ROS downstream of GPVI consists of two distinct phases: an initial Syk-independent burst followed by additional Syk-dependent generation. In this review, we will discuss recent findings on the regulation of platelet function by ROS, focusing on GPVI-dependent platelet activation and thrombus formation.

1. Introduction

As natural by-products of aerobic metabolism, reactive oxygen species (ROS) are comprised of radical and non-radical oxygen species formed by the partial reduction of oxygen, including superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂), and hydroxyl radical (HO•) [1]. ROS are generated endogenously in response to stimulation by cytokines, xenobiotics and bacterial invasion as well as during mitochondrial oxidative metabolism [2]. Abnormal elevation of ROS is associated with oxidative stress, and implicated in diseases such as atherosclerosis [3], diabetes [4] and, neurodegeneration [5] as well as in aging [6], resulting in potential damage to proteins, lipids and nucleic acids.

In recent years, there is increasing evidence demonstrating that ROS also regulate cellular signaling pathways involved in physiological and pathological processes [7,8]. One potential mechanism for how ROS regulate signaling pathways is through oxidation of cysteine (Cys) residues on signaling proteins [9]. ROS also regulate the function of

anucleate blood platelets [10–12], and the use of antioxidants in the prevention and treatment of thrombotic or cardiovascular diseases has been investigated [13,14]. One important pathway for generating intracellular ROS in human platelets is through ligand binding to the platelet collagen receptor, glycoprotein (GP)VI [15]. In this review, we will discuss recent findings on the role of ROS in regulating GPVI-dependent platelet activation and thrombus formation.

2. Platelet adhesion, activation and thrombus formation

At sites of vascular injury, platelets roll, adhere and firmly attach to subendothelial matrix by platelet primary membrane receptors, glycoprotein (GP)VI which binds collagen, and GPIb α , the major ligand-binding subunit of GPIb-IX-V complex, which binds von Willebrand factor (VWF) and other ligands, through recognition of exposed VWF/collagen in the damaged blood vessel wall, initiating platelet adhesion and triggering rapid activation [16,17]. Activation of signaling

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pathways lead to cytoskeletal rearrangements, shape change and activation of the platelet integrin, α IIb β 3, from a low- to a high-affinity state which binds ligands including fibrinogen, VWF, fibronectin and vitronectin [18,19]. Activated platelets secrete platelet agonists, such as adenosine diphosphate (ADP) and thromboxane A2 (TXA2) which bind to purinergic (P2Y) receptors and thromboxane receptor (TP), respectively, to reinforce α IIb β 3-dependent platelet aggregation [19]. Further, along with a number of other cell types, platelets express surface CD40 ligand (CD40L; also known as CD154) a molecule that is crucial for cell signaling in both adaptive and innate immunity. On activated platelets, CD40L can be cleaved by metalloproteinases into a soluble form [20]. The majority (> 95%) of plasma soluble CD40L (sCD40L) is derived from platelets [20] and sCD40L enhances platelet activation in an auto-amplification loop [21], leading to heightened platelet aggregation and platelet-leukocyte interactions. These interactions have been implicated in the onset of atherothrombosis [22,23] and arterial hypertension [24]. This process involves signaling via tissue necrosis factor- α receptor associated factor (TRAF) 6, which is emerging as a molecular target that can be therapeutically modulated in a range of disorders [25,26]. Furthermore, activated platelets also promote coagulation by surface expression of phosphatidylserine (PS) and release of procoagulant factors that lead to thrombin generation and fibrin formation. In particular, GPVI and GPIb-IX-V are crucial in the initiation of platelet thrombus formation under abnormal pathological shear stress and altered blood flows, for example within stenosed arteries, and also play an important role in initiating thrombus formation in experimental models of cerebral vascular stroke [27–30].

3. Platelet GPVI: expression, shedding and generation of intracellular ROS

GPVI is a type I transmembrane receptor and a member of the immunoglobulin (Ig)-like superfamily, only expressed on platelets and megakaryocytes, and consisting of two extracellular Ig domains, a short mucin-like domain, a transmembrane domain and a cytoplasmic tail [31]. The major physiological ligand for platelet GPVI is collagen, although laminin and fibrin have also been identified as GPVI ligands [32–35]. In addition, several nonphysiological ligands have been described, including cross-linked collagen-related peptide (CRP) [36], snake toxins (convulxin, alborhagin, crotarhagin) [37–39] and anti-GPVI antibodies [40]. The GPVI cytoplasmic domain contains binding sites for calmodulin (CaM) via a membrane-proximal positively-charged sequence [41,42], and for Src family kinases (Fyn and Lyn) via a proline-rich sequence and involved in GPVI-dependent signal transduction [43]. GPVI is also co-associated with the Fc receptor γ -chain (FcR γ), required for GPVI surface expression [31,36]. Binding of multivalent ligands and cross-linking of GPVI/FcR γ leads to phosphorylation of an immunoreceptor tyrosine-based activation motif (ITAM) within the cytoplasmic tail of FcR γ by the Src family kinase, Lyn, in turn resulting in recruitment and assembly of spleen tyrosine kinase (Syk) and subsequent activation of various adaptor proteins, such as 76-kDa tyrosine phosphoprotein (SLP-76) and Linker-for-activation of T cells (LAT), eventually resulting in the activation of phospholipase C γ 2 (PLC γ 2). PLC γ 2 causes elevation of cytosolic Ca²⁺ and leads to activation of integrin α IIb β 3 [44,45].

Another important consequence of ligand binding to GPVI is dissociation of CaM from the cytoplasmic tail of GPVI, and subsequent metalloproteinase (ADAM10)-mediated GPVI ectodomain shedding, generating a soluble extracellular fragment (sGPVI) and a membrane-associated remnant fragment [46–48]. In human plasma, sGPVI represents a platelet-specific marker of platelet function, and is elevated in athero-thrombotic, inflammatory and immune-related disorders. Interestingly, ligand binding to GPVI also causes rapid transient disulfide-dependent receptor dimerization through oxidation of the Cys residue in the GPVI cytoplasmic tail [49], suggesting an oxidative submembranous environment in activated platelets [50,51].

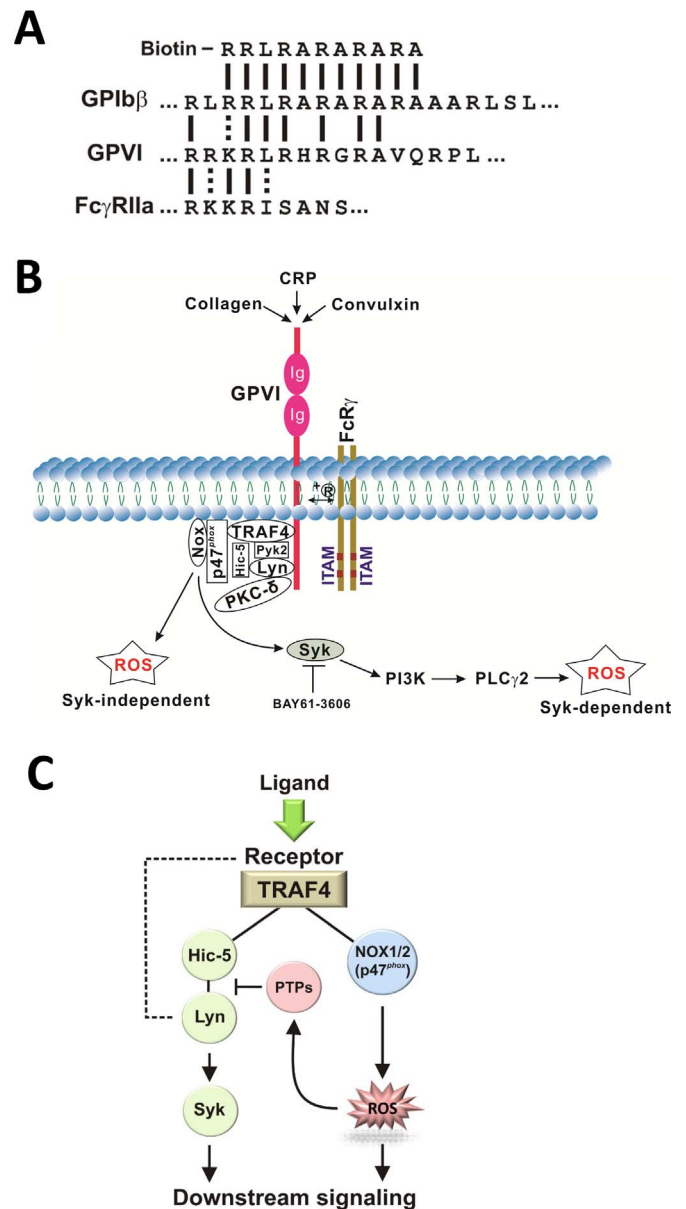


Fig. 1. (A) Calmodulin-binding sequences in platelet receptors. Positively-charged lysine (K)- and arginine (R)-rich calmodulin-binding sequences of GPIb β , GPVI and Fc γ RIIa, and the biotinylated amino acid sequence used to identify TRAF4 as a potential receptor-binding partner (solid lines indicate identical residues, dotted line conservative substitutions). (B) ROS generation downstream of GPVI signaling. TRAF4 and its binding partner p47^{phox} (NOX1/2 subunit) are constitutively associated with the cytoplasmic tail of GPVI, providing a link of ROS generation to downstream signaling in response to ligand binding (such as CRP, collagen or convulxin) via Src family kinase Lyn, which is constitutively bound to GPVI as well as to TRAF4-binding partners Pyk2 and Hic-5, and PKC- δ , which is associated with Lyn, resulting in phosphorylation of PKC- δ . Once activated, PKC- δ regulates p47^{phox} phosphorylation, translocation and NOX activation. Two distinct Syk-independent and Syk-dependent phases of ROS generation occur after GPVI ligation, distinguishable using a Syk inhibitor such as BAY61-3606. (C) Overview of potential link between platelet receptors, TRAF4 and redox complexes. TRAF4 directly associated with the cytoplasmic region of GPVI or other receptors could link these receptors to Lyn (via Hic-5) and NOX (via p47^{phox}), thereby activating downstream Syk-dependent or Syk-independent pathways leading to platelet aggregation. ROS may also activate cytosolic protein tyrosine phosphatase (PTPs) to negatively regulate Hic-5/Lyn. Hic-5 can also bind Pyk2 (panel B). Phosphorylated Lyn is also directly associated with GPVI (dashed line). See the text for details.

Healthy human platelets contain basal levels of intracellular ROS, however these levels rapidly increase following GPVI stimulation [10,52–55]. Unique evidence for the mechanism by which engagement

of GPVI may generate oxidative stress was provided in recent studies identifying tumor necrosis factor receptor associated factor (TRAF4) as a novel binding partner for GPVI (Fig. 1A), using a biotinylated amino acid sequence based on calmodulin-binding sequences of GPVI, GPIIb β and Fc γ RIIIa [56]. TRAF4 selectively binds to the cytoplasmic tail of GPVI and interacts with p47^{phox}, a subunit of the NADPH oxidase 1 and 2 (NOX1/2) complex, which is the major source of ROS production in platelets. In addition, TRAF4 also interacts with other signaling proteins such as Pyk2 and Hic-5, which are constitutively associated with Lyn, a key component of the GPVI-dependent signaling pathway. In platelets, Lyn is associated with protein kinase C (PKC) δ and phosphorylates PKC- δ in human platelets stimulated with the GPVI agonist, convulxin [57]. As PKC- δ is required for p47^{phox} phosphorylation and subsequent translocation to the membrane [58], TRAF4 directly associated with the cytoplasmic region of GPVI (or another receptor) could link Lyn (via Hic-5 and Pyk2), PKC- δ (via Lyn) and NOX1/2 (via p47^{phox}) upon GPVI engagement. This would trigger p47^{phox} phosphorylation and subsequent activation of NOX1/2, thereby activating downstream Syk-dependent or Syk-independent pathways leading to platelet aggregation (Fig. 1B). Intraplatelet ROS may also activate cytosolic protein tyrosine phosphatases (PTPs) to negatively regulate Hic-5/Lyn (Fig. 1C). For example, ROS can reversibly inactivate phosphorylation-dependent signaling by oxidation of an active site cysteine in PTPs such as SHP-2 [12,59], allowing Lyn-dependent signaling to occur. Deficiency of peroxiredoxin II, an antioxidant enzyme, enhanced GPVI-mediated platelet activation, possibly through the defective elimination of H₂O₂ and/or impaired protection of SHP-2 against oxidative inactivation, leading to increased tyrosine phosphorylation of key components for Syk-dependent pathway of GPVI signaling [60]. Peroxiredoxin II-deficient platelets also displayed increased adhesion/aggregation upon collagen stimulation. These interactions involving both redox and tyrosine phosphorylation pathways provide a mechanism for rapid initiation of downstream signaling by platelets in response to ligands acting at GPIIb-IX-V/GPVI (VWF/collagen). Interestingly, given the common binding sequence on GPVI for both TRAF4 and calmodulin (Fig. 1A), it is not yet proven whether binding of these proteins to this site is mutually exclusive. However, if calmodulin competes with TRAF binding, then dissociation of calmodulin promoting receptor shedding could provide a mechanism to limit ligand-induced GPVI-dependent ROS production via TRAF4/NOX. Further studies are needed to test this possibility.

There is abundant evidence supporting the importance of platelet ROS in platelet function and in human health and disease. Higher levels of platelet ROS are associated with thrombotic diseases, hypertension, diabetes, hypercholesterolemia and metabolic syndrome [61]. ROS may also be involved in other pathways controlling platelet activation [62]. Catalase-induced reduction of cytosolic H₂O₂ impaired platelet aggregation [63]. Platelets treated with NOX inhibitors (see below), or platelets from patients with X-linked chronic granulomatous disease that are genetically deficient in the catalytic NOX subunit gp91^{phox}, also exhibited decreased intracellular Ca²⁺ mobilization and platelet activation [64]. In addition, ROS scavengers or inhibitors of O₂^{•-} production, apocynin and catalase, attenuated platelet activation, whereas ROS donors such as 2,3-dimethoxy-1,4-naphthoquinone (DNMQ) enhanced platelet activation [64]. Furthermore, a recent study has demonstrated a role for isoprostanes, produced by oxidation of arachidonic acid, in platelet activation and thrombus growth [64], whereas pharmacologic antioxidants, such as vitamin C, vitamin E or transresveratrol, can inhibit platelet activation and aggregation [11].

4. Potential therapeutic targeting of ROS in platelets

Targeting components of ROS generation including subunits of NOX1/2 in platelets is an appealing strategy, although this requires increased understanding of precise mechanisms, as global targeting of NOX activity for example could have potentially deleterious effects in

cells other than platelets. Improved targeting could involve selective blockade of receptor-localizing elements of NOX complexes, thereby inhibiting ligand-induced ROS production by platelet-specific receptors such as GPIIb-IX-V or GPVI/Fc γ R, and/or enabling the uncoupling of ROS burst following platelet exposure to VWF/collagen essential for thrombus formation at high arterial shear rates.

Inhibition of NOX activity using NOX inhibitors, diphenyleneiodoniumchloride (DPI) and apocynin, significantly impeded platelet activation, aggregation and thrombus formation [10,53]. There are 7 NOX family members (NOX1-5 and DUOX1-2) identified in mammalian cells [65–67]. Expression of both NOX1 and NOX2 has been confirmed in both human and mouse platelets [68,69]. Studies using a pharmacological inhibitor specific for NOX1, ML171 (2-acetylphenothiazine) as well as NOX2-deficient mice [70] have shown that NOX1, but not NOX2, is responsible for GPVI-dependent ROS production and TXA₂ generation, mediated in part through p38 MAP kinase-dependent signaling. Both NOX1 and NOX2 are required for collagen-mediated thrombus formation at arterial shear. Other evidence suggests that NOX1 is selectively important in G protein coupled receptor-mediated platelet activation induced by thrombin, but is not required for GPVI-dependent platelet activation induced by CRP in mice [69]. Furthermore, NOX2^{-/-} platelets displayed reduced ROS generation and Ca²⁺ mobilization during GPVI-dependent platelet activation. Additional selective inhibitors and accounting for likely differences between human and mouse platelets are required to define the relative role of NOX1/2 in GPVI-dependent ROS production under different pathophysiological conditions.

GPVI-dependent generation of intracellular ROS in human platelets measured using the redox-sensitive fluorescent dye, H2DCF-DA, was not affected by inhibitors of α IIB β 3 (abciximab, GRGDSP peptide) or Src family kinases (PP1, PP2), but strongly inhibited by an inhibitor of NOX (apocynin) [15]. Inhibitors of PI3K (LY294002) or Syk (piceatannol, BAY61-3606) partially inhibited ROS generation. In this regard, approximately one-third of total GPVI-dependent ROS occurred within 2 min (ROS_{initial}), without further ROS generation in the presence of BAY61-3606 or piceatannol, suggesting the Syk-dependent pathways are not required for the initial burst of ROS, but are involved in subsequent ROS generation, and consistent with an initial Syk-independent burst and an additional Syk-dependent phases of GPVI-dependent ROS generation (Fig. 1B). Consistently, two distinct phases of ROS formation were also observed in analyses of single mitochondria ROS production, termed ROS-induced ROS release (RIRR), representing the initial or triggering phase followed by a subsequent amplification of ROS release [71]. In addition, RIRR in smooth muscle cells was also reported to be via NOX activation [72]. Moreover, in angiotensin-II treated endothelial cells, ROS generation resulting from angiotensin-II-mediated NOX activation decreased the mitochondrial membrane potential, leading to mitochondrial ROS formation and subsequent activation of PKC, and enhanced activation of NOX, resulting in increased intracellular ROS production [73]. Further supporting the role of mitochondrial ROS in NOX activation, angiotensin-II-induced activation of NOX was reduced in vascular smooth muscle cells where mitochondrial function had been ablated [74]. Mitochondrial ROS also induces NOX activation in human leukocytes and increased NOX activity was observed in mice with mitochondrial superoxide dismutase deficiency [75]. Taken together, these studies suggest a functionally relevant crosstalk between NOX and mitochondria exist [76–78]. However, whether mitochondria play a significant role in the subsequent ROS generation in GPVI-stimulated platelets remains unclear and requires further investigation.

5. Conclusion

Engagement of primary platelet receptors, GPIIb-IX-V and GPVI, that initiate thrombus formation at arterial shear rates leads to a rapid increase in intracellular ROS above basal levels, and is a key step in

platelet activation following exposure to physiological ligands, VWF/collagen. Targeting linkage of these or other receptors to ROS-generating NOX1/2 complexes via the adaptor, TRAF4, could provide far greater selective inhibition of ROS generation/platelet reactivity than general antioxidant treatment. Understanding the basic mechanisms underpinning receptor-linked ROS in human platelets is critical, and could ultimately enable maintenance of optimal platelet intracellular ROS levels in disease states associated with exacerbated thrombotic propensity.

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