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# 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 (FcRγ) 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 submembranous environment in platelets after GPVI stimulation. Using a redox-sensitive fluorescent dye ($H_2$DCF-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 ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$), 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 GPIbα, 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, αIIbβ3, 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 αIIbβ3-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 (FcRγ), required for GPVI surface expression [31,36]. Binding of multivalent ligands and cross-linking of GPVI/FcRγ leads to phosphorylation of an immunoreceptor tyrosine-based activation motif (ITAM) within the cytoplasmic tail of FcRγ 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 Cγ2 (PLCγ2). PLCγ2 causes elevation of cytosolic $Ca^{2+}$ and leads to activation of integrin αIIbβ3 [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].

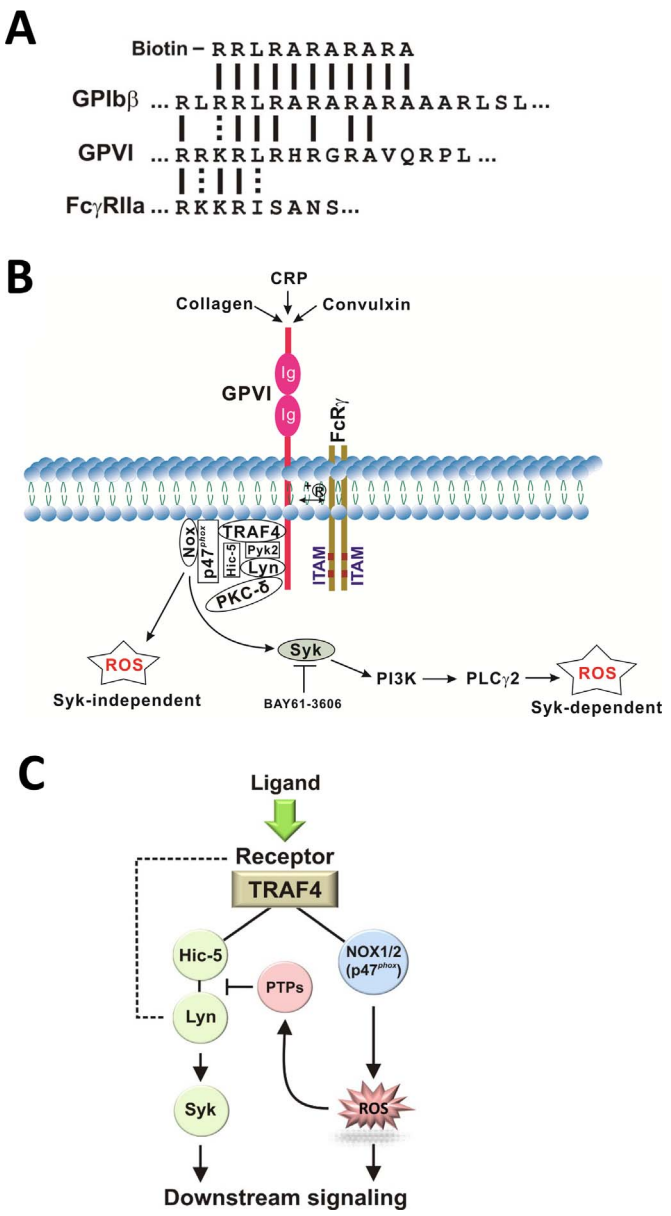


**Fig. 1. (A) Calmodulin-binding sequences in platelet receptors.** Positively-charged lysine (K)- and arginine (R)-rich calmodulin-binding sequences of GPIbβ, GPVI and FcγRIIa, and the biotinylated amino acid sequence used to identify TRAF4 as a potential receptor-binding partner (solid lines indicate 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