Integrin alphaIIbbeta3-dependent Reactive Oxygen Species Production in Platelets

BY

ZHENG XU
Beijing Normal University, 2012

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Master of Science in Pharmacology
in the Graduate College of the
University of Illinois at Chicago, 2016

Chicago, Illinois

Defense Committee:

Xiaoping Du, Chair and Advisor

Richard Minshall

Marcelo Bonini

ACKNOWLEDGEMENTS

I would like to thank for all the people who had supported this study and provided their generous help all along the way.

Sincere thanks are given to my advisor, Dr. Xiaoping Du, who had offered me the chance to work in this outstanding team, and provided his support and guidance throughout my time studying there. Dr. Du, as a brilliant scientist, an excellent mentor, nurtured critical thinking and rigorous attitude in me, which are fundamental qualities it takes to be a researcher. I value all the precious lessons learnt throughout time that would definitely continue to guide my future career and life.

I want to express special thanks to my research committee members, Dr. Richard Minshall and Dr. Marcelo Bonini, for their enlightening suggestions, guidance, and being available to enable frequent communications. Their contributions to this work are invaluable and indispensable.

I would like to thank our collaborators who had contributed to the study. Thanks Dr. M. Ushio-Fukai and Dr. Karl-Heinz Krause (University of Geneva) for kindly providing transgenic mice. Thanks for Peter. C. Hart and Chengxia He from Bonini Lab, for sharing their knowledge and resources pertaining key experiments.

I would like to thank all the previous and current members from Du Lab, Dr. Keegan M. Delaney, Aleksandra Stojanovic-Terpo, whose pioneer work explored and built the foundation for this study, Dr. Bo Shen and Estevez Brian, who had advised me numerously regarding study design and experimental skills, Claire Chang, Dr. Cheng Ni, Dr. Aiming Pang, Dr. Yujie Cui and Dr.

Yanyan Bai, who had been working with me side by side on daily basis and offered kind and generous helps, without which none of these works could have been accomplished.

Special gratitude is given to Dr. Patricia W. Finn, Dr. David Perkins and Dr. John O'Bryan, for offering their warm heart, kindness, and graciousness to me when in predicament.

I am honored and grateful to have been working in Department of Pharmacology, along with all the fellow students, faculties and staffs, who had not only exchanged research ideas with me constantly, but also made my life here both joyful and fulfilled.

Thanks for my family and friends, for having been supporting me, loving me, and being there with me all the time.

TABLE OF CONTENTS

CHAPTER PAGE

LIST OF FIGURES

FIGURE PAGE

LIST OF ABBREVIATIONS

ACD	acid-citrate-dextrose
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic 3’, 5’-adenosine monophosphate
Carboxy-H2DCF	6-carboxy-2',7'-dichlorodihydrofluorescein diacetate
CRP	collagen related peptide
cGMP	cyclic guanine monophosphate
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DTS	dense tubular system
EDTA	ethlenediaminetetraacetic acid
FcRγ	Fc receptor gamma
FcγRIIA	Fc gamma receptor IIA
FITC	fluorescein isothiocyanate
GC	guanylyl cyclase
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GPVI	glycoprotein VI
GPIbα	glycoprotein Ib alpha
GPIbβ	glycoprotein Ib beta
GPIb-IX-V	glycoprotein Ib-IX-V complex
GPIX	glycoprotein IX
GPV	glycoprotein V
H2DCF	dichlorodihydrofluorescein diacetate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSC	hematopoetic stem cells
ITAM	immunoreceptor tyrosine kinase activation motif
IP3	inositol 1,4,5-triphosphate
LAD	leukocyte adhesion deficiency
LPS	lipopolysaccharide
NaHCO3	sodium bicarbonate
NO	nitric oxide
MAPK	mitogen-activated protein kinase
MnCl2	manganese chloride
MPs	microparticles
OCS	open canalicular system
PAGE	polyacrylamide gel electrophoresis
PAR	proteinase-activated receptor
PBS	phosphate-buffered saline
PF4	platelet factor 4
PGE1	prostaglandin E1

LIST OF ABBREVIATIONS (continued)

PI3K	phosphoinositide-3 kinase
PKA	cAMP-dependent protein kinase
PKB/Akt	protein kinase B
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PLCγ2	phospholipase Cγ2
PS	phosphatidylserine
PVDF	polyvinylidene fluoride
RIAM	Rap1-GTP-interacting adaptor molecule
ROCK	Rho-dependent kinase
SDS	sodium dodecyl sulfate
Ser	Serine
SFK	Src family protein kinase
TXA2	thromboxane A2
vWF	von Willebrand factor

SUMMARY

Excessive Reactive Oxygen Species (ROS) is associated with all known cardiovascular risk factors and is believed to play an important role for the onset of cardiovascular diseases. As a family of multisubunit enzymes complexes, NADPH oxidases deliberately generate superoxide and/or hydrogen peroxide. Evidence shows that NADPH oxidase are the key generators of ROS in the blood vessel wall and other tissues during cardiovascular disease progression. Studies from both human and animal platelet has suggested the role of NADPH-dependent ROS in platelet activation and thrombosis. However, the mechanism of how ROS production is regulated during platelet activation, and the effect of NOX-dependent ROS on platelet function remains unclear.

This study focuses on illustrating the mechanism of how does integrin αIIbβ3 regulate ROS production and NOX activity in platelets. Integrin outside-in signaling is shown to be important for ROS production and NOX2 activation. Data here demonstrated P47phox phosphorylation and ROS production could be induced by integrin-ligation without need for other stimuli, and it is augmented by outside-in signaling activator, MnCl2, while disrupted by selective outside-in inhibitor, mP6. And the absence of β3 is shown to almost completely abolished p47phox activation, even in CRP-stimulated platelets.

In addition, it has demonstrated that NOX1 and NOX2 knockout platelets was defect in stable adhesion, which is not further inhibited by mp6. This could possibly suggest ROS reversely inhibits integrin outside-in signaling. However, platelet spreading is not affected by NOX knockout. Along with previous data showing that NOX1/2 knockout mainly affected

platelet granule secretion, and second wave of platelet aggregation, which requires integrin outside-in signaling (Delaney et al. 2016), we concluded NOX-dependent ROS is important for integrin outside-in signaling and its functional consequences.

Altogether, this study, provided insight as how ROS production in activated platelets is regulated, forming a theoretical foundation for new antithrombotic strategy basing on intervening integrin outside-in pathway-regulated NOX activity.

I. LITERATURE REVIEW

1 Introduction

Platelets are small anucleate cellular fragments that circulate in the blood. They are the smallest type of circulating blood cells, and have a characteristic discoid shape. Their distinctive appearance was originally described as “small plates,” hence the name platelets (Osler 1886). They are derived from precursor megakaryocytes in a process called thrombopoiesis. Mature human platelets have a physiological count of 1.5 to 4 x 10^8/mL. Platelets typically circulate in the blood for 7-10 days. After 7-10 days of lifespan in the circulation, they are cleared by the spleen and liver. About 1×10^{11} newly produced platelets are supplied each day to replace the cleared platelets (Kumar and Clark 2012). In mice, commonly used laboratory species, circulating platelet count is much higher, at approximately 1-2 x 10^9/mL, with a shorter lifespan of around 5 days(Fox 2007). In non-mammalian vertebrates such as zebrafish, platelets are nucleated and are named thrombocytes (Ma et al. 2011). Platelets play an essential role in processes such as physiological hemostasis and pathological bleeding and thrombosis. Quantitative or qualitative disorders of platelets lead to increased bleeding tendency in patients such as Glanzmann thrombasthenia and Bernard - Soulier syndrome (Alexander and Landwehr 1949, Gautier and Guinand-Doniol 1952, Caen et al. 1971, Nurden and Caen 1974). Platelets/thrombocytes also contribute to other physiological and pathological functions beyond hemostasis (Ware, Corken, and Khetpal 2013) At the site with ruptured atherosclerotic plaques in arteries, platelets are recruited to form platelet-rich thrombi, which can cause acute thrombotic events such as stroke and heart attack.

2 Platelets

2.1 Platelets Origin and Morphology

Derived from megakaryocytes, Platelets and thrombocytes are differentiated from hematopoetic stem cells (HSC), which mainly reside in the bone marrow in mammals, or kidney and thymus in zebrafish (White 1988, Hartwig 1992). The polyploid megakaryocytes (50–100 μm) are the source of platelets. Mature megakaryocytes contain multiple nuclei, massive amounts of granules and proteins, which are fragmented into individual platelets during platelet genesis (Italiano et al. 1999). To assemble and release platelets, megakaryocytes become polyploid by endomitosis (DNA replication without cell division) and then undergo a maturation process in which the bulk of their cytoplasm is packaged into multiple long processes called proplatelets, and the nucleus is extruded. Platelet formation can be divided into two phases: first phase takes up to days and is featured by megakaryocyte maturation and development to complete and requires megakaryocyte-specific growth factors. Megakaryocytes undergo rapid nuclear proliferation and enlargement of cytoplasm, they during which are filled with cytoskeletal proteins, platelet-specific granules and abundant membrane materials to prepare for the platelet assembly process. The second phase is relatively quick and usually be completed in hours. During this phase, megakaryocytes remodel their cytoplasm into proplatelets and then preplateles. A megakaryocyte may extend 10–20 proplatelets (Richardson et al. 2005). The preplatelets then fission to generate discoid platelets. It takes ~5d in human and 2-3d in rodents from megakaryocyte polyploidization, maturation to release platelets (Ebbe et al. 1965, Odell and Jackson 1968, Odell, Jackson, and Friday 1970).

Although the size of platelets could be variable between individuals, their mean diameter is of 1-3 µm, mean thickness of 0.5 µm, a mean cell volume of 6 to 10 fL.

2.2 Platelets Structure

2.2.1 Membranes

Similar to that of other cell types, platelet plasma membrane is a lipid bilayer, which contains transmembrane receptors, mediating important platelet functions such as adhesion, spreading and aggregation. Surface-connected open canalicular system (OCS) is a specialized membrane structure of platelets that distinct from other cell types. The OCS derives from the platelet plasma membrane, and consists of invaginations of the surface membrane that form channels that tunnel into the cytoplasm (Escolar and White 1991). The channels of the OCS serve to transport various chemicals, molecules, and constituents in the blood to enter the deepest recesses of the cell (White 1972). The OCS also functions as a source for increased surface area, where the membrane may be accessed to greatly expanding the total surface area in spreading platelets.

Dense tubular system (DTS) of platelets serves as smooth endoplasmic reticular system in other cell type, which exists as predominant calcium reservoir (Daimon and Gotoh 1982). Upon binding of inositol 1,4,5-triphosphate (IP3), product of Phospholipase C (PLC), to receptors on the DTS membrane, calcium is released from DTS (Sage and Rink 1987). This facilitates platelets shape change, and the centralization and concomitant secretion of granules (Robblee, Shepro, and Belamarich 1973, Menashi, Davis, and Crawford 1982) .

2.2.2 Cytoskeleton

The discoid shape of resting platelet is maintained by a highly specialized cytoskeleton, which contributes to platelet shape and integrity under high shear forces during circulation. The major cytoskeletal components of the resting platelet are the actin, tubulin and spectrin etc.

One of the distinguishing feature of platelet cytoskeleton system is marginal microtubule coils (White and Krivit 1967, White 1968), which supports platelet's characteristic disc shape. Firstly characterized by Jim White, the microtubule ring was described as approximately 100μm long, winding 8 to 12 times under plasma membrane (White 1968). Platelets microtubule is long, hollow cylinders assembled from α and β tubulin dimers, which are in constant equilibrium of assembly and disassembly. In resting platelets polymers and dimers approximately make up equal fractions of tubulins. Among several isoforms of β tubulins, β1 was shown to be the dominant isoform of tubulin that is specific to megakaryocytes and platelets (Schwer et al. 2001). Drugs that disassemble microtubules, such as vincristine, colchincin, ornocodazole destroy platelets morphology causing them to lose the round shape (White 1968). β1-tubulin (-/-) mice have thrombocytopenia (of 50% of normal) resulting from defective platelets genesis. Their platelets also lack the discoid shape and microtubule coils at the periphery, containing only 2 or 3 coils (Schwer et al. 2001).

Similar to tubulin, actin is in an assembly and disassembly equilibrium. About 60% of the actin subunits remain in storage, which can convert to filaments when platelets are activated. As platelets express high concentrations of actin cross-linking proteins, including filamin (Feng and Walsh 2004) and α-actinin (Rosenberg, Stracher, and Burridge 1981) that connect the platelet filaments to form rigid cytoplasmic network. Interaction between filamins and the

cytoplasmic tail of the GPIbα subunit of the GPIb-IX-V complex plays essential role to structural organization in resting platelets. This interaction anchors filamin's self-association domain and partner proteins at the plasma membrane, and aligns GPIb-IX-V complexes into rows on the surface of platelets. Megakaryocytes that lack this filamins and GPIbα interaction forms large and fragile platelets which are low in numbers.

Spectrin in another integral part featuring platelets membrane structure. Specifically, spectrin strand binds actin filament through interconnecting with filamins, forming triangular pores covering the plasma membrane and OCS. The network recruits signaling molecules during platelet activation. Proteins associated with the cytoskeleton function to cross-link actin filaments, bind signaling molecules, and interact with membrane proteins (Fox 2001).

2.2.3 Granules

Platelets contain three major recognized granules, defined by their unique molecular content: α-granules, dense granules and lysosomes. The most abundant are α-granules, with about 40 per platelets. These granules have a diameter about 200 to 500 nm, are spherical in shape with dark cores (Sixma, Slot, and Geuze 1989). They contain proteins essential for platelet adhesion, some of them are synthesized by the megakaryocytes, like platelet factor 4 (PF4), coagulation factor V, thrombospondin, von Willebrand Factor (vWF), others are acquired by the uptake and packaging of plasma proteins via endocytosis and pinocytosis (de Larouziere et al. 1998), such as fibrinogen. Several membrane proteins that are critical to platelet functions are also packed in α-granules, including αIIbβ3, P-selectin, CD36. Dense granules are around 250nm, fewer in numbers than α-granules (3 to 8 per platelets), and identified by their electron-opaque dense cores. The contents of dense granule are primarily intended to recruit

additional platelets to the site of vascular injury. They contain a variety of substances, such as the adenine nucleotides, ATP and ADP, calcium, other platelet agonists such as serotonin, and catecholamines. Platelets also contain few lysosomes, usually 0 or 1 per platelet. The acidified organelles are round in shape and slightly smaller than α-granules, containing various hydrolases. Platelets lysosomes may serve as endosomal digestion compartment, but their physiological roles in hemostasis is not well understood.

2.3 **Platelets Function**

Platelets are specialized circulating cells that play central role in hemostasis and thrombosis. The attributes acquired by platelets through evolution reflect their ability to response to injuries of vessel wall and sustain repeated contact with normal vessel wall without premature activation. Two major functions of platelets upon vascular injury are to form primary thrombus and facilitate coagulation. In addition, platelets participate in the innate immune defense, act as modulators of the inflammatory response. Pathologic thrombus formation occurs when platelets activation overwhelms their protective mechanisms. Platelets form a thrombus at sites of ruptured atherosclerotic plaques, generating thrombi present locally or distant emboli, thus trigger life threatening events such as angina, heart attack and stroke.

Platelet plug formation started in more than one way. In the event of trauma to the vessel wall, circulating platelet are exposed to subendothelial matrix via adhesive receptors, captured and then activated by adhesive proteins that are not normally present in blood, such as collagen and vWF multimers. Platelets rapidly generate initial monolayer that supports subsequent thrombin formation and recruitment of additional platelets. A key to this

interaction is the presence of receptors integrin α2β1 and Glycoprotein VI (GPIV) that can bind with collagen, and GPIb (to a lesser extent αIIbβ3) that can bind with VWF. In the setting of thrombotic or inflammatory disorders, platelets can be activated by thrombin, which binds protease-activated receptor (PAR), family member of G protein-coupled receptors (GPCRs).

The initial monolayer is insufficient to prevent bleeding, but it started signaling cascade leading to stable platelet adhesion, aggregation and thrombus formation. Local accumulation of components released from granules are strong agonists to stimulate platelet aggregation. The dense bodies of platelets contain important secondary agonists like ADP or serotonin. ADP binds to two receptors on the platelets surface. The P2Y1-receptor mobilizes Ca2 + and stimulates shape change (Fabre et al. 1999) , while he P2Y12-receptor potentiates platelet secretion (Dorsam and Kunapuli 2004). Serotonin (5-hydroxytryptamine, 5-HT) binds to the Gq-coupled 5HT2A-receptor and amplifies platelet response. Thromboxane A_2 (TxA_2) are synthesized from arachidonate in platelets, diffused outward to activate other platelets, thus amplifying the initial response. The central receptor mediates accumulation of platelets into a hemostatic plug is integrin αIIbβ3 (GPIIb/IIIa). Activated αIIbβ3 binds to multivalent ligands such as fibrinogen, fibrin and VWF, linking activated platelets together that results in growth of thrombus (Li, Delaney, et al. 2010).

In addition, platelets contribute to blood coagulation by facilitating thrombin generation from prothrombin, and the deposition of insoluble fibrin to the site of injury. Upon activation, the platelet plasma membrane externalizes high amounts of phosphatidylserine (PS) from the inner leaflet of lipid bilayer. PS is also highly exposed on microparticles (MPs) that shed from platelets plasma membrane. Those processes greatly increase catalytic surface for

procoagulation by enabling thrombin generation and Ca^{2+}-dependent assembly of blood clotting factors.

2.4 Platelets Receptors and Signaling

2.4.1 Integrin

Integrins are important family of transmembrane adhesion receptors that mediate cell adhesion to the extracellular matrix proteins, or cell-cell interaction. Integrins are usually classified according to their respective β subunits. Different family members are displayed on platelets and other cell types, among them αIIbβ3 is the predominant type (fibrinogen receptor) (Wagner et al. 1996), αVβ3 is vitronectin receptor), α2β1 the collagen receptor, α5β1 the fibronectin receptor, and α6β1 the laminin receptor. Integrin αMβ2 (Mac-1) and αLβ2 (LFA-1) are the major family members of leukocyte adhesion proteins (Kishimoto et al. 1987).

Glanzmann's thrombasthenia is a rare bleeding disorder caused by integrin αIIbβ3 deficiency or mutations. Compared to the Bernard–Soulier syndrome, patients have increased bleeding tendency, but normal platelet counts and normal size (Ginsberg et al. 1983).

Leukocyte adhesion deficiency I (LAD I) syndromes are derived from mutations on β2 subunit that impair expression or function of leukocyte integrins. Subjects with LAD II are intact in integrin expression and function but defect in glycosylation of ligands for the selectin family or glycosylation of other glycoconjugates. LAD syndromes are failures of innate host defenses against microorganisms that result from defective tethering, adhesion, and targeting of myeloid leukocytes to sites of microbial invasion (Bunting et al. 2002).

2.4.1.1 Integrin Structure

In total, research in the last decades identified 24 canonical integrins formed from 18 α integrins and 8 β integrins in mammals (Shattil, Kim, and Ginsberg 2010). Each α integrin subunit could pair with one of the β integrins to form an integrin heterodimer complex, which mediates cell adhesion to a wide range of extracellular matrix proteins, non-matrix proteins, or other cells.

αIIbβ3 is the most abundant intergrin heterodimer on platelets with an average of 80,000 copies per cell (Wagner et al. 1996) . In most integrins the amino-terminal domain, β-propeller and the βA domain, non-covalently bind together to form a 'head'. βA domain forms the main ligand-binding site, as does αA domain in integrins other than αIIbβ3. Crystal structures revealed that low-affinity state integrins are in a bent conformation with their ligand binding sites placing in proximity to the membrane surface (Shattil, Kim, and Ginsberg 2010). Intermediate extended conformations with a closed head corresponds to activated state integrin, where the conformational changes in the extracellular domain lead to increased ligand affinity. Binding of ligand induces further conformational changes, resulting in a high-affinity extended open conformations that fully activate integrin signalling downstream (Choi et al. 2013).

2.4.1.2 Integrin Inside-out Signaling

The distinct feature of integrins is that they transduce signal bidirectionally (Hynes 2002). Binding of intracellular proteins like talin and kindlin to the tail domain induces changes in the extracellular ligand binding domain of integrins to activated state, this is so-called 'inside-out' signaling (Calderwood et al. 1999, Ma et al. 2008). Talin is a large (280-kDa), cytoplasmic

protein, its translocation to plasma membrane is promoted by Ras family small GTPase Rap1 and Rap1-GTP-interacting adaptor molecule (RIAM) (Lee et al. 2009, Chrzanowska-Wodnicka et al. 2005). CalDAG-GEF1 stimulates the activity of the Rap1b, by converting it from the guanosine diphosphate (GDP)- to the activated guanosine triphosphate (GTP)-bound form. Once active, Rap1 bound to GTP interacts with the adaptor RIAM to promote the association of talin with β subunit. Talin binds NPLY motif on integrin β cytoplasmic tails, disrupting the edogenous interaction beween the α and β cytoplasmic tails that constrains integrin in the closed conformation. Direct talin has been recognized as a common final step in integrin activation (Tadokoro et al. 2003). The kindlin family, particularly kindlin-3 in platelets, was identified to be an important regulator of integrin activation. Deletion of kindlin-3 in platelets results in increased bleeding, defective platelet aggregation and integrin activation (Malinin et al. 2009, Moser et al. 2008). It was hypothesized that kindlin acts as a co-activator during integrin activation by binding to the β cytoplasmic tail at C-terminal NXXY motif (Harburger, Bouaouina, and Calderwood 2009). On the other hand, data suggested kindlins have little direct effect on integrin αIIbβ3 affinity but increase multivalent ligand binding by promoting the clustering of talin-activated integrins (Ye et al. 2013). It is noteworthy that talin and kindlin are also playing roles in integrin outside-in signaling. Evidences show integrins activation by talin is required for fibrin clot retraction (Haling et al. 2011), and kindlin is required for integrin outside-in signaling to enable firm adhesion and spreading (Montanez et al. 2008).

2.4.1.3 Integrin Outside-in Signaling

Ligand binding of integrin to extracellular matrix initiates series of intracellular signaling, resulting in responses like calcium mobilization, protein phosphorylation, and cytoskeletal

remodeling, this is called 'outside-in' signaling (Hynes 2002). Platelet integrin αIIbβ3 outside-in signaling leads to platelet spreading, retraction, granule secretion, stable adhesion, second wave of platelet aggregation, therefore playing central roles in both thrombosis and hemostasis (Li, Delaney, et al. 2010, Shattil and Newman 2004).

Gong et al found that heterotrimeric guanine nucleotide-binding protein (G protein) Gα13 directly bound to the integrin β3 cytoplasmic domain which was promoted by integrin αIIbβ3 ligand binding and guanosine triphosphate (GTP) loading of Gα13 (Gong et al. 2010). Shen et al has recently shown that Gα13 and talin bind to mutually exclusive but distinct sites within the cytoplasmic domain of integrin β3. Furthermore, it was also shown that Gα13 and talin bind to β3 in opposing waves (Shen et al. 2013). After talin binds to β3 to mediate inside-out signaling as previously discussed, it is dissociated from β3 stimulated by integrin-ligand binding. This dissociation allows Gα13 bind to an ExE motif within the β3 tail. Gα13-β3 interaction induces outside-in signaling through the phosphorylation and activation of the Src Family Kinase (SFK), c-Src. Src is primed for activation by direct interaction with an integrin beta tail, and integrin clustering stabilizes activated Src by inducing intermolecular autophosphorylation on Tyr418 in the c-Src activation loop (Arias-Salgado et al. 2003). This direct interaction is mediated by the SH3 domain of the c-Src, and the C-terminal ^{759}RGT motif in integrin β3 (Flevaris et al. 2007). In the early phase of outside-in signaling, c-Src activates RhoGAPs (Rho GTPase Activating Proteins), such as p190RhoGAP, promoting RhoA-GTP converting to RhoA-GDP (Arthur and Burridge 2001).Therefore, Src inhibits the RhoA-dependent retractile signaling pathway, leading to cell spreading. Rho GTPases, Rho, Rac and Cdc42 play key role in the cell spreading signals: Rho regulates stress fiber and focal adhesion

assembly, Rac regulates the formation of lamellipodia protrusions and membrane ruffles, and Cdc42 triggers filopodial extensions at the cell periphery (Hall 2005). In contrast, migrating cells concurrently display retraction of membrane at their trailing edge. Coordination of these two opposite membrane movement is important for cell migration (Huttenlocher and Horwitz 2011). Study shows integrin signaling induces phosphorylation on c- Src ^{747}Tyr/^{759}Tyr, leading to elevated intracellular calcium level and activation of calpain (calcium-dependent, non-lysosomal cysteine proteases) (Fox et al. 1993). In the late-phase of outside-in signaling, calpain cleaves the cytoplasmic domain of β3 integrin at ^{759}Tyr after its dephosphorylation, which releases the inhibitory effect of the Gα13/Src pathway thereby promotes RhoA-dependent platelet retraction. (Du et al. 1995, Xi et al. 2003, Xi et al. 2006). Activated RhoA in turn stimulates the activation of Rho dependent kinase (ROCK) to phosphorylate and inactivate myosin light chain (MLC) phosphatase, providing an increase in MLC phosphorylation and consequent actin-myosin mediated retraction (Kimura et al. 1996). In conclusion, calpain cleavage of integrin β3 at ^{759}Tyr switches the functional outcome of integrin signaling from cell spreading to retraction (Flevaris et al. 2007).

Moreover, Src phosphorylate β3 on cytoplasmic tyrosine, this phosphorylation results in the physical linkage of αIIbβ3 to the cytoskeleton (Jenkins et al. 1998). Finally, Src promote the activation of Syk and its incorporation into the integrin signaling complex (Boylan et al. 2008). Phosphorylation of Fc gamma receptor IIA (FcγRIIA) promotes ITAM-dependent signal transduction. Syk facilitates the activation of PLCγ2 and platelet activation similar to the ITAM signaling pathway stimulated by GPVI (Woodside et al. 2001, Abtahian et al. 2006) .

2.4.2 The Glycoprotein Ib-IX-V Complex (GPIb-IX-V)

Under high shear rate flow conditions, which are often seen in the arteries and capillaries, adhesion receptor GPIb-IX-V binding to vWF is critical for platelet adhesion to the site of vascular injuries. In addition GPIb-IX-V also transmits signals leading to platelet activation, aggregation and secretion. Various cellular signal pathways contribute to regulation of GPIb-IX-V (Du 2007). Deficient or mutation of GPIb-IX-V results in the Bernard-Soulier syndrome, a bleeding disorder characterized by giant platelets and defective interaction with von Willebrand factor (Lopez et al. 1998). Pseudo-von Willebrand disease results from gain-of-function mutations (Gly233 or Met239 to Val) that cause spontaneous vWF binding and bleeding associated with subsequent platelet depletion (Andrews, Lopez, and Berndt 1997, Berndt et al. 2001, Lopez et al. 1998). A mouse with Bernard–Soulier-syndrome expresses a fusion protein containing transmembrane/cytoplasmic domains of GPIb and extracytoplasmic domain of IL4-receptor ameliorates the large-platelet phenotype, suggesting GPIb–IX–V, especially transmembrane/cytoplasmic domain could regulate platelet size. Combined blockade of GPIb–IX–V and αIIb3 simultaneously achieved significant anti-thrombotic effect in baboon arterial-stenosis models, while didn't increase bleeding-time which is usually caused by clinical αIIb3-blockade alone (Wu et al. 2002).

2.4.2.1 Structure

GPIb-IX-V complex is composed of four subunits: GPIbα, GPIbβ, GPIX and GPV. They are expressed in the complex in a ratio of 2:2:2:1 (Andrews, Lopez, and Berndt 1997, Berndt et al. 2001, Bodnar et al. 1999). GPIbα (CD42bα) is linked to GPIbβ (CD42b/CD42c) with disulfide bond to form GPIb, and GPIbαβ are non-covalently associated with GPIX (CD42a) and GPV

(CD42d) in a ratio of 1:1. It was suggested that the GPIb-IX complex is sufficient for both ligand-binding and signaling functions (Du 2007). The four subunits are all members of the leucine-rich repeat (LRR) superfamily, as they have LRRs of ~24 amino acids that are flanked by conserved N- and C-terminal disulfide loop structures (Lopez 1994). Another subunit, GPV is loosely associated with GPIb-IX in a ratio of 1:2. There has been report showing a subset of GPIb-IX-V constitutively associates with the lipid rafts in unstimulated platelets and the remaining GPIb-IX-V are further recruited to these lipid raft region upon stimulation with VWF (Shrimpton et al. 2002).

GPIbα is the largest subunit in the complex, with 610 residues and the molecular weight of 135KDa (Canobbio, Balduini, and Torti 2004), composed of N-terminal domain, transmembrane region and cytoplasmic tail (Lopez et al. 1987). 282 amino acids make up N-terminal domain, which contains 8 LRRs that serve as binding site for various GPIb-IX-V ligand, such as VWF, thrombin, P-selectin, αMβ2, coagulation factors Xl/Xlla, and high molecular weight kininogen (Canobbio, Balduini, and Torti 2004). N-terminal domain is followed by residues 283-302 that enriched in negatively charged residues, including 3 sulfated tyrosines (Dong, Li, and Lopez 1994). Following them is a long and highly glycosylated mucin-like macroglycopeptide domain (residue 303-485) that connects to single transmembrane region (residues 486-514). The cytoplasmic tail of GPIbα (residues 515-610) contains binding site for intracellular molecules that's important for the regulation of VWF-induced platelet adhesion and activation (Du, Fox, and Pei 1996, Andrews and Fox 1992). The characteristic tandem LRRs is essential for normal processing and function of GPIb-IX-V (Ulsemer et al. 2000). LRRs 2, 3, and 4 of GPIbα are required for VWF binding (Shen et al. 2002), mutations within the 2, 5, 6, 7 LLRs

have been found associated with Bernard-Soulier syndrome (Whisstock et al. 2002). The three tyrosine residues, Tyr276, Tyr278, Tyr279, in the negatively charged region is also demonstrated to be important for VWF binding (Marchese et al. 1995). The cytoplasmic tail of GPIbα is essential in signal transduction activity of GPIb, since it contains binding site for multiple intracellular molecules, such as 14-3-3ξ (Du, Fox, and Pei 1996), proteins of cytoskeleton, such as filamin (Andrews and Fox 1992). Binding with these proteins are recognized as important for regulation of VWF-induced GPIb-IX-V binding and signaling.

GPIbβ has a single LRR flanked with conserved sequence, with 181 residues and the molecular weight of 25KDa (Lopez et al. 1988). It is covalently lined to GPIbα through disulphide bond and interact noncovalently with GPIX through residues 15-32 (Lopez et al. 1988, Kenny, Morateck, and Montgomery 2002). The 34 residues-long cytoplasmic domain has been found to interact with calmodulin (Andrews et al. 2001), 14-3-3ξ (Andrews et al. 1998), and contains Ser166 that can be phosphorylated by cAMP-dependent protein kinase (PKA) (Wardell et al. 1989).

GPIX that is 22KDa in weight and consists of 160 residues has only a single LRR with conserved flanking sequence, similar to GPIbβ (Hickey, Williams, and Roth 1989), but it contains a short cytoplasmic tail with only 5 amino acids. GPV is a 82kDa protein consisting of 544 residues, with 15 LRRs in its extracellular domain and 16 amino acids composed cytoplasmic tail (Hickey et al. 1993). It directly interacts with GPIbα subunit (Li et al. 1995) to form the complex. GPV has been shown to associates with calmodulin and 14-3-3ξ through its cytoplasmic domain (Andrews et al. 2001, Andrews et al. 1998). It's suggested GPV can strengthen binding of VWF and GPIb-IX-V under high shear condition (Fredrickson et al. 1998). In addition, data also

showed GPV binds collagen (Moog et al. 2001) and form high-affinity binding site for thrombin(Dong, Sae-Tung, and Lopez 1997). However, another group reported cleavage of GPV by thrombin promotes GPIb-IX mediated platelet activation (Ramakrishnan et al. 2001), making the exact role of GPV remained unclear.

2.4.2.2 Functions

While integrin-mediated platelet adhesion can occur at low shear flow conditions, GPIb-IX-V interaction with VWF is essential for platelet adhesion under high shear rate flow, like those occurring in arterioles and small arteries (Bergmeier et al. 2006). Moreover, after binding VWF, GPIb-IX-V initiated signaling cascade that culminate in platelet integrin activation, leading to stable platelet adhesion, aggregation and thrombus formation. Patients lack either the receptor (Bernard-Soulier syndrome) or the ligand (von Willebrand disease) suffer from severe bleeding disorders, providing evidences for the essential role of GPIb-IX-V and VWF interaction in normal hemostasis.

Under pathological conditions like thrombotic thrombocytopenic purpura (TTP), a multimeric form of von Willebrand factor (VWf) is found in the plasma that are derived from the deficiency of a divalent cation-activated, VWf-cleaving metalloprotease. This deficiency causes systemic platelet aggregation under the high-shear conditions of the microcirculation, due to spontaneous binding of GPIb-IX-V to uncleaved ultra-large VWF (ULVWF) (Moake 2002). In sepsis, a systemic inflammatory response, microvascular thrombosis and consumptive thrombocytopenia contributes to high mortality of the disease (Hotchkiss and Karl 2003). Bacteria endotoxin (lipopolysaccharide, LPS) targets endothelium during inflammation, causing the endothelial cells to release multimeric VWF from their Weibel-Palade bodies. VWF attached

on endothelial cells recruits circulating platelets, leading to enhanced thrombogenicity at inflammatory foci (Schorer, Moldow, and Rick 1987). Studies applying antibodies that block GPIb-IX-V and VWF interaction suggested involvement of GPIb in endotoxin-induced responses (Katayama et al. 2000). Furthermore, GPIb was identified as the platelet counterreceptor of Mac-1 on leukocytes, therefore facilitating firm adhesion and transplatelet migration of leukocytes on vascular thrombus at the site of inflammation (Simon et al. 2000). Collectively, GPIb is recognized as a key player mediating the interactions between platelets, leukocytes, and endothelium in thrombosis and inflammation.

2.4.2.2.1 VWF Binding Function

VWF binding function of GPIb-IX-V is related to the shear rate of blood flow. The velocity of blood flow is maximal in the center of blood vessel and falls to close to zero at the wall. Thus, the gradient of velocity, or shear rate is maximal at the vessel wall, decreasing towards the center (Ruggeri 1997, Kroll et al. 1996). In vivo, shear rates are much higher in small arterioles of 10–50 μm diameter, where the shear rates are estimated to vary between 470 and 4700 s^{-1}. When atherosclerotic plaques forms and partially occludes arteries, shear rates of 11,000 s^{-1} have been calculated(Back, Radbill, and Crawford 1977). Studies have shown that at shear rates >1000 s^{-1}, platelet adhesion is strongly dependent on VWF.

As a multimeric protein synthesized by megakaryocytes and endothelial cells, VWF is stored in their α-granules and Weibel-Palade bodies, respectively, from where it can be released upon cell activation. It also circulates in plasma as dimer and function as a carrier for factor VIII. Mature VWF contains 2050 amino acid residues, with molecular weight of about 250kDa, and characterized to have A, B, C and D structural domains. A1 is the domain on VWF

that mediates interaction with GPIb but it requires conformational change to increase its affinity (Miyata et al. 1996). The conformational change occurs when 1) VWF is immobilized to extracellular matrix or 2) it subjects to high shear stress (Siedlecki et al. 1996). Three different regions on GPIbα involves interaction with VWF A1 domain: 1) the N-terminal flanking sequence and the LRR region (Shen et al. 2000, Cauwenberghs et al. 2001), 2) the C-terminal flanking domain (Shen et al. 2002), and 3) highly negative-charged region (Marchese et al. 1995, Ward et al. 1996). Under experimental conditions, nonphysiological modulators like botrocetin (snake venom toxin) and ristocetin (a bacterial antibiotic glycopeptide) can binds to different sites within the protein, mimicking the collagen-induced changes in VWF which activate its binding activity with GPIb-IX-V (Berndt et al. 1992, Lopez 1994, Ware 1998).

When secreted into plasma, ULVWF undergoes proteolysis by ADAMTS 13 (A Disintegrinlike And Metalloprotease with ThromboSpondin motif). Cleavage by ADAMTS 13 generates VWF in smaller size that is less adhesive. Deficient activity of ADAMTS 13 (either congenital or autoantibody-mediated) leads to large multimers of plasma VWF, which spontaneously binds with GPIb-IX. This results in enhanced platelet aggregation, microvascular thrombosis and consumptive thrombocytopenia in TTP (Lammle, Kremer Hovinga, and Alberio 2005).

2.4.2.2.2 14-3-3ξ in Regulation of VWF Binding Function

Aside from aforementioned VWF conformation change, intracellular signaling molecules, such as 14-3-3ξ, are also important modulators of VWF binding activity of GPIb-IX-V. 14-3-3ξ forms homodimer and serves as central intracellular hub for many signal transduction pathways, primarily through binding phosphoserine and phosphothreonine (Xiao et al. 1995). A

14-3-3ξ homodimer has two binding sites for GPIb. It recognizes S(606)GHSL(610) on the cytoplasmic tail of GPIbα, only when Ser(609) is phosphorylated (Bodnar et al. 1999, Du, Fox, and Pei 1996). Additional binding sites includes Arg557-Gly575 and Leu580-Ser590 on GPIbα (Andrews et al. 1998, Mangin et al. 2004), and Ser166 on GPIbβ (Andrews et al. 1998). Therefore, VWF can potentially bind to both GPIbα and GPIbβ at the same time, or GPIbα alone. A 'toggle switch' model was proposed to illustrate the mechanism of 14-3-3ξ regulating VWF binding function of GPIb-IX-V (Fig.1) (Dai et al. 2005, Du 2007).

In this model, increase of cAMP concentration results in activation of protein kinase A (PKA). PKA phosphorylates GPIbβ on site Ser166, which induces binding of 14-3-3ξ simultaneously to GPIbα and GPIbβ. GPIb in this state is 'switch off' and has low-affinity for VWF. When intracellular cAMP decreases, 14-3-3ξ dissociates from GPIbβ while remaining binding to GPIbα, results in active state of GPIbα. Disrupting 14-3-3ξ interaction with GPIbα, by either mutagenesis or inhibitory peptides, abolishes VWF binding function of GPIb (Dai et al. 2005).

2.4.2.3 GPIb-IX-V Mediated Signaling Pathways

VWF binding of GPIb-IX-V initiates a intracellular signaling cascade that activates integrin inside-out pathway and thus stable platelet adhesion, aggregation and secretion (Du 2007). Various intracellular secondary mediators have been shown to involve in this process, including increase of intracellular calcium, activation of protein kinase C (PKC) (Kroll et al. 1991), synthesis of thromboxane A2 (Lagarde 1988), activation of protein kinase G (PKG) (Li et al. 2003), and phosphoinositide 3-kinase (PI3-K) (Yap et al. 2002).

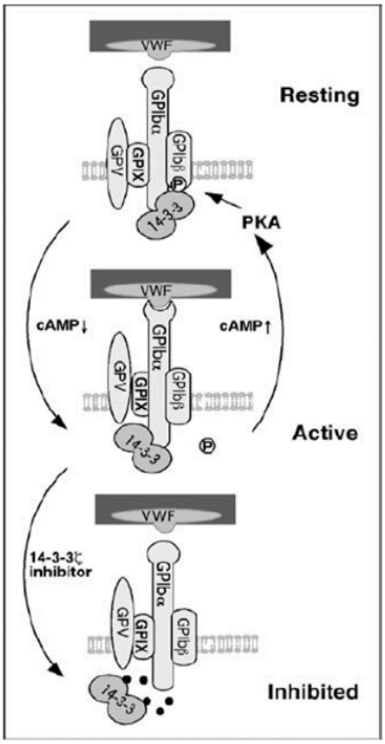


Figure 1. **A 'toggle switch' model of glycoprotein Ib–IX–V (GPIb–IX–V) regulation.** Increases in intracellular cAMP induce GPIbb phosphorylation, allowing 14–3–3z interaction with both GPIba and GPIbb, locking GPIb–IX in an 'off' or 'resting' state. A decrease in cAMP dissociates 14–3–3z from GPIbb and thus 'switches on' the von Willebrand factor (VWF) binding function. Inhibition of 14–3–3z binding to GPIba disrupts the 'toggle switch', inhibiting the VWF binding function of GPIb–IX–V. PKA, protein kinase A. (Reprinted with permission from Curr Opin Hematol.)

The immediate interactors downstream of GPIb-IX-V are suggested to be Src Family Kinase (SFK) and SFK-dependent PI3-K/Akt pathway. SFK inhibitors blocked all responses downstream of GPIb-IX-V under static or flow conditions (Kasirer-Friede et al. 2004). Using

mouse platelets deficient in Lyn, Src, a study by Liu et al has demonstrated botrocetin/VWF-mediated TxA2 production is dependent on signaling initiated by Lyn and enhanced by Src (Liu et al. 2005). Moreover, immunoprecipitation studies demonstrated that Src and Lyn form complex with GPIb-IX and PI3-K upon VWF binding. This complex formation requires presence of p85, the regulatory subunit of PI3-K (Wu et al. 2003). 14-3-3ξ is also indicated to promote the association of GPIb-IX and PI3-K, possibly by recruting molecules to same subcellular compartment and form complex (Munday, Berndt, and Mitchell 2000).

Akt family of protein kinases are involved in downstream of PI3-K signaling pathway, with Akt1 and Akt2 being the members expressed in both human and mouse platelets. They are recruited by phosphoinositide-dependent protein kinase (PDK) to the membrane, where they are phosphorylated and activated (Kandel and Hay 1999, Lawlor and Alessi 2001, Kroner, Eybrechts, and Akkerman 2000, Chen et al. 2004, Woulfe et al. 2004). In Src and Lyn knockout mice, reduction of Botrocetin/VWF-induced Akt phosphorylation in platelets has also been reported (Liu et al. 2005). Furthermore, PI3-K/Akt pathway stimulates platelet NO synthesis by activating nitric-oxide synthase 3 (eNOS), leading to activation of soluble guanylyl cyclase (GC) elevation, and thus elevation of cGMP (Stojanovic et al. 2006). GPIb-IX-V mediated platelet activation, secretion, integrin activation are shown to require participation of cGMP-dependent protein kinase (PKG) (Li et al. 2003, Li et al. 2004, Li, Xi, and Du 2001). Following studies by our lab illustrated a sequential activation of p38 and Erk mitogen-activated protein kinase (MAPKs) downstream of PKG pathway, which ultimately initiates inside-out signaling of integrin (Li et al. 2006). Collectively, these data delineated a SFK-PI3K-Akt-sGC-cGMP-PKG-p38-Erk signal transduction pathway induced by agonist binding of GPIb-IX-V.

2.4.3 Collagen Receptors

Collagens are important subendothelial adhesive ligands that can activate platelets through binding to their corresponding receptors. There are two major collagen receptors on platelets, integrin α2β1 and GPVI, with the former one responsible for adhesion and platelet anchoring, and the latter pricipally mediating the signaling transduction that leads to platelet activation (Clemetson et al. 1999, Clemetson and Clemetson 2001, Nieswandt and Watson 2003).

GPVI is the major established platelet receptor for collagen, and was shown to be responsible for collagen-induced platelet activation. Its importance was first recognized in an patient who has autoantibodies against GPVI. The patient's platelets are defective in collagen-induced aggregation (Sugiyama et al. 1987). GPVI contains two extracellular Ig C2 loops that binds to collagen, a stalk consisting of mucin-like domains, a transmembrane region and a short cytoplasmic tail (Clemetson and Clemetson 2001). It belongs to immunoglobulin (Ig) superfamily, and noncovalently associates with FcRγ chain to mediate platelet activation signaling (Gibbins et al. 1997). Upon interaction with its ligands, multiple GPIVs cluster and crosslink, resulting in tyrosine phosphorylation within ITAM domain of FcRγ. This process has been shown to require SFK family members, Lyn and Fyn, and is regulated by tyrosine phosphatase CD148 (Ezumi et al. 1998, Quek et al. 2000, Senis et al. 2009). Phosphorylation at ITAM domain of FcRγ recruits tyrosine kinase Syk, which initiates a cascade of signaling events (Yanaga et al. 1995). Downstream targets of Syk includes transmembrane adapter linker for activated T cells (LAT) and Src homology 2 domain– containing leukocyte phosphoprotein of 76-kDa (SLP-76). This further recruits Gads, Bruton tyrosine kinase (Btk), PI3-K, and phospholipase

C gamma (PLCγ) 2 to form a complex (Watson et al. 2005, Nieswandt and Watson 2003, Watson et al. 2001, Quek, Bolen, and Watson 1998, Pasquet et al. 1999, Watanabe et al. 2003). Activation of PLCγ2 transduces signal further through inositol 1,4,5-trisphosphate (IP3), diacyglycerol (DAG) and PKC pathway, leading to Ca2+ mobilization, TxA2 synthesis, granule secretion and integrin activation (Fig.2) (Li, Delaney, et al. 2010) .

Another major adhesion receptor of collagen on platelets is integrin α2β1. Cre/loxP-mediated β1 knockout platelets showed only mild defect on fibrillar collagen-induced aggregation. Compared with complete defect of platelets lacking GPIV, these data demonstrated GPVI plays the central role in platelet collagen interactions, while integrin α2β1 strengthens adhesion but is not essential (Nieswandt et al. 2001). On the otherhand, platelets isolated from α2 integrin subunit knockout mice failed to adhere to type I collagen under either static or shear-stress conditions (Chen et al. 2002). Nieswandt et al also reported that α2β1 have a complementary function in ex vivo thrombus formation, facilitating the central function of GPVI in the platelet activation (Kuijpers et al. 2003). Thus, although α2β1 may has some facilitating function in collagen-induced platelet activation, its exact role still remain contraversial. Contradicted conclusions are likely due to the different experimental settings employed.

2.4.4 Thrombin Receptors

Thrombin is able to induce platelet activation at concentration as low as 0.1nM, making it the most potent activator of platelets and critical for maintaining normal hemostasis. Platelets respond thrombin stimulation through member of protease-activated receptor (PAR) family, which includes PAR1, PAR3 and PAR4. PAR1 and PAR4 are the receptors account for

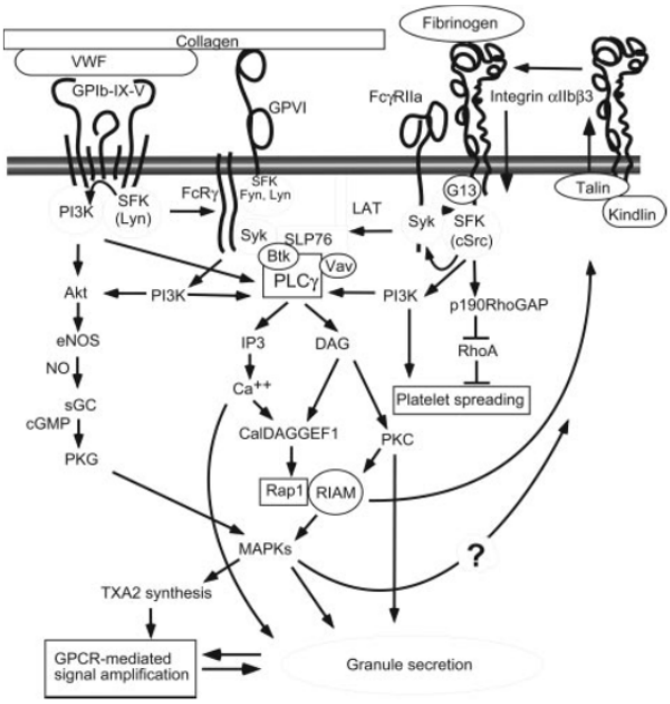


Figure 2. Signaling pathways of major platelet adhesion receptors.
(Reprinted with permission from ATVB)

activation of platelets by thrombin in human, while PAR3 and PAR4 are the ones present on mouse platelets (Kahn et al. 1999, Kahn et al. 1998). PAR2 is a trypin/tryptase/matriptase receptor present on other cell types such as endothelium, neutrophils and epithelial cells and smooth muscle cells (Nystedt et al. 1994, Frungieri et al. 2002, Seitz et al. 2007).

Thrombin cleaves the N-terminal extracellular domain at a specific site of the receptor to expose a new N-terminus as tethered ligands (Vu et al. 1991, Rasmussen et al. 1991). PAR1 is a high-affinity receptor to thrombin than PAR4, thus believed to be the major receptor for thrombin under physiological conditions especially at low agonist concentration. Activation of PAR4 requires 10 to 100 fold higher concentrations of thrombin than are required for PAR1, probably due to its lacking of the hirudin-like sequences that facilitates interaction with thrombin (Xu et al. 1998, Ishii et al. 1995). Studies demonstrated peptides for either receptor

was sufficient to activate platelets (Vu et al. 1991, Kahn et al. 1998). However, inhibition of PAR1 by antagonist, antibody or desensitization abolished platelet activation induced by low dose thrombin but only impaired it partially at high dose. In contrast, inhibition of PAR4 alone had little effect at either dose. Finally, blockade of both receptors at the same time ablated platelet activation even with high dose thrombin (Kahn et al. 1999). These observations suggested PAR1 is responsible for platelet response to low dose thrombin, while PAR4 readily contributes to platelet activation at high dose thrombin. Kinetic studies suggested calcium mobilization responding to thrombin in platelets could be separated into two discrete components, of which PAR1 induces a rapid spike response, followed by a slower prolonged response from PAR4 (Covic, Gresser, and Kuliopulos 2000). Thus, PAR4 serves to extend the duration of high intracellular calcium levels that maintains the late phase of platelet aggregation process. PAR3 and PAR4 also play different roles on mouse platelets. Depletion or inhibition of PAR3 on mouse platelet ablated platelet activation at low but not high concentration of thrombin. A model suggested PAR3 does not mediate signaling independently but rather facilitates PAR4 function as a cofactor for receptor cleavage by thrombin (Nakanishi-Matsui et al. 2000). Although PAR family accounts for most thrombin signaling in platelets, other receptors might also play complementary roles. One example is GPIbα, which binds thrombin with its Asp(272)-Glu(282) segment (De Cristofaro et al. 2000). Blocking this thrombin-binding site on GPIbα results in impaired platelet activation induced by alpha-thrombin (De Marco et al. 1991). This is likely due to a ‘cofactor’ function of GPIbα, since evidences showed that its interaction with thrombin enhances thrombin cleavage of PAR-1 on platelets (De Candia et al. 2001). A recent study in our lab proposed a new model for the role of

GPIbα in thrombin-induced PAR signaling: PAR-dependent platelet activation induced by low dose thrombin requires cooperativity of GPIbα, and conversely, thrombin-induced GPIbα-dependent signaling also requires cooperativity of PAR (Estevez et al. 2015). This mutually dependent cooperativity model may shed new light to the controversity.

The PARs couples to intracellular heterotrimeric guanosine nucleotide-binding proteins (G-proteins) to pass signaling to downstream. PAR1 and PAR4 in human platelets couple to G12/13 and Gq (Coughlin 2005, Russo, Soh, and Trejo 2009). Gα12/13 activated by PAR1 and PAR4 initiates sigaling through p115RhoGEF/RhoA/ROCK-dependent pathway, resulting in phosphorylation and inhibition myosin light chain phosphatase (MLCP). In turn, this process causes increased phosphorylation of myosin light chain, thus contraction of actin that results in rapid platelet shape change and degranulation (Klages et al. 1999). In addition, PARs induce strong activation of Gq, whose downstream target includes phospholipase C-β (PLC- β) .PLC-β hydrolyzes phosphatidylinositol-4,5-diphosphate (PIP2) to produce IP3 and DAG, which in turn stimulates calcium mobilization and activation of PKC that is important contributor for intergrin activation and granule release (Hers et al. 1998, Walker and Watson 1993). PARs do not directly activates Gi, instead they trigger ADP release from granules that activates Giβγ coupled P2Y12 receptor (stated in the next section) (Kim et al. 2002).

2.4.5 Purinergic Receptors

Purine and pyramidine nucleotides, like ADP and ATP are important platelet aggregation agents that are released from damaged cells and released from platelets dense granules. ADP and ATP can binds to P2Y receptors on platelets. P2Y1 and P2Y12 are GPCR that coupled with

Gq and Gi, respectively. ATP also binds to cation-permeable ligand gated ion channels P2X1 (Wang et al. 2003).

Once binds to ligand, P2Y1 receptor activates Gαq that is couple with it, which mediates activation of PLC, production of IP3 and DAG, activation of PKC and subsequently transient mobilization of calcium (Fabre et al. 1999, Murugappa and Kunapuli 2006). Early studies using ADP antagonists, such as adenosine-2', 5'-diphosphate (A2P5P) and adenosine-3', 5'-diphosphate (A3P5P), specifically inhibits platelet shape change and aggregation but not affects inhibition of adenylyl cyclase (Daniel et al. 1998, Savi et al. 1998). A patient with severe deficiency of ADP-induced platelet aggregation, however, has normal P2Y1 receptor (Leon, Vial, et al. 1999). In addition, P2Y1 receptor null mouse platelets do not have shape change and aggregates with ADP stimulation, however, are intact with ability to inhibit adenylyl cyclase (Leon, Hechler, et al. 1999, Fabre et al. 1999). P2Y12 receptor couples to Gαi, mediates inhibition of of adenylyl cyclase and amplifies platelet aggregation (Nessel 2005). A patient with congenital bleeding disorder that fails to respond to ADP was shown to be deficient in P2Y12 receptor (Hollopeter et al. 2001). P2Y12 receptor null mouse platelets fail to inhibit adenylyl cyclase and exhibit prolonged bleeding times, impaired response to ADP, reduced sensitivity to thrombin and collagen. However, these platelets are unaffected in terms of shape change and calcium flux (Foster et al. 2001). P2Y1 and P2Y12 are both required for normal aggreation, since specific inhibition of signaling through either receptor was sufficient to block ADP-induced platelet aggregation (Jin and Kunapuli 1998). What's more, ADP was reportecd to promote integrin-dependent spreading on immobilized fibrinogen, and apyrase, an ADP scavenger, is shown to reduce extend of platelet spreading (Haimovich et al. 1993, Jirouskova, Jaiswal, and

Coller 2007). In mice that lack Gi2, the predominant platelet Gi subtype, ADP-dependent platelet aggregation and integrin αIIbβ3 activation were strongly reduced (Jantzen et al. 2001). These data suggest the role of ADP for integrin activation, platelet spreading and aggregation.

Another P2 receptor on platelets is P2X1, which is activated by ATP only and belongs to ligand-gated ion channels (Mahaut-Smith et al. 2000). The function of P2X1 was not recognized until Rolf et al. used a selective P2X1 receptor agonist, α,β-methylene-ATP (α,β-MeATP) to show it could evoke a transient shape change in human platelets (Rolf, Brearley, and Mahaut-Smith 2001). However, following studies revealed stimulation of P2X1 induced shorter filopodia than ADP stimulation, centralizing secretory granules. (Rolf and Mahaut-Smith 2002). Although P2X1 could positively regulates early platelet response to low-dose collagen, promotes aggregation, fast reversible shape change and degranulation, P2X1 activation alone is insufficient to stimulate platelet aggregation and full platelet activation (Toth-Zsamboki et al. 2003, Oury et al. 2001, Hechler et al. 2003, Takano et al. 1999). Thus, P2X1 is considered to play a role synergizing other platelet activation pathways.

2.4.6 Thromboxane Receptors

Upon activated by agonists, such as thrombin, ADP, collagen, platelet release arachidonic acid stored in the plasma membrane in the form of phospholipid. The enzyme cyclooxygenase (COX) catalyzes the reaction to produce prostaglandin endoperoxide PGH2, which is an intermediate then converted to TxA2 by thromboxane synthase (Crofford 1997, Dubois et al. 1998). Aspirin is the most wildly known antithrombotic drug that inhibits COX, thus the production of TxA2. Once formed, TxA2 diffuses through the plasma membrane, acts through autocrine and paracrine manners, activates and recruits additional platelets to the site

of thrombus (Svensson, Hamberg, and Samuelsson 1976). There are two subtypes of thromboxane prostanoid (TP receptors) on human platelets, TPα and TPβ, as the result of alternative splicing of the primary transcript (Dorn 1989, Takahara et al. 1990, Narumiya et al. 1993). Boths receptors belong to GPCR family, and the typical form, TPα, is coupled to G12/13 and Gq protein (Djellas et al. 1999, Knezevic, Borg, and Le Breton 1993).

3 NADPH Oxidases

3.1 Reactive Oxygen Species

Reactive Oxygen Species (ROS) are chemically unstable molecules that were historically recognized as byproducts of biological reactions. However, gradually increasing studies have shown that at physiological level, ROS can act as intracellular signaling molecules, regulating several important pathways (Sugamura and Keaney 2011). The oxidation chain reaction primarily starts from production of superoxide (O2-), which are generated from redox components such as those form mitochondrial electron transport chain, or through catalization of enzymes like NADPH Oxidase (NOX), xanthine-oxidase etc. (Cadenas and Davies 2000, Babior 1999). Two molecules of superoxide react to generate hydrogen peroxide (H2O2). This reaction is known as dismutation and catalyzed by enzyme named superoxide dismutase (SOD). H2O2 and superoxide can also react to generate hydroxyl radicals (OH•) in the presence of iron. Due to their impaired electron in the outer orbital, O2- and OH• are very reactive chemical entities that tends to gain an additional electron to form stable non-radical state. In contrast, H2O2 are more properly pro-oxidant non-radical agents, thus less reactive. Other species occur in different biological system, such as hypochlorous acid (HOCl) that formed in neutrophils from H2O2 and chloride, singlet oxygen (Δ^1O_2) that formed at the site of inflammation from oxygen,

and ozone (O_3) that generated from singlet oxygen, catalyzed by antibodies (Kanofsky 1989, Wentworth et al. 2002).

ROS readily react with a great number of biological molecules including proteins, nucleic acids, lipids, carbohydrates and inorganic molecules. Oxidation of these targets may lead to irreversible damage or loss of function. Due to this reason, ROS have be widely linked with the aging process (Beckman and Ames 1998). An important beneficial function of ROS is examplified in host defense. In neutrophils and macrophages, NOX deliberately generates massive amount of ROS upon exposure to microorganisms or inflammatory mediators. This so-called 'respiratory burst ' is a key measure for the microbicidal activity of phagocytes.

3.2 NADPH Oxidase

3.2.1 Structure and Function

NADPH oxidase are classified into three groups, based on the consistence of structural domains in each member (Lambeth et al. 2000). Among the NOX family, NOX2, also known phogocyte NADPH oxidase (phox), is the prototype and has been most thoroughly studied. Structural studies had provided evidence about six transmembrane domains of gp91phox of which the COOH and NH2 terminus are facing the cytoplastic side. This feature is shared by all NOX family members. There are two histidines on transmembrain domain III and IV, spanning tw hemes that act as part of electron transport chain. On the COOH terminus, exist a flavin adenine dinucleotide (FAD) and a NADPH binding domain. As single electron transporter, NOX captures electron from NADPH and pass it along to FAD, then hemes, finally oxygen on the extracellular side of the membrane.

Activation of NOX depends on a serie of translocation and assembly of different subunits upon stimulation. The catalytic subunit, a.k.a. gp91phox constitutively associates with the enzyme stablizing component, p22phox, both of them are membrane-associated subunits. In the absence of p22phox, pg91phox is unstable. This is reported in the patient with CGD. (Dinauer et al. 1990, Parkos et al. 1989). The activation of NOX is regulated by the assembly of cytosolic subunits p47phox, p40phox, p67phox and the small GTPase RAC with gp91phox and p22phox on the membrane. Signalling triggers including protein kinase C and AKT phosphorylate p47phox, the organizer subunit, at its autoinhibitory region (AIR). Serine residues in this region become extensively phosphorylated, up to as many as nine sites. Among them, Ser303/304 are two residues that are of special interest because alanine double mutant of Ser303/304 almost completely abolished NOX activity (Inanami et al. 1998, Hoyal et al. 2003). Another study reported single mutation of Ser304 compromised the oxidase activity to more than 50% (Faust et al. 1995). Phorsphorylation of AIR will release bis-SRC-homology 3 (bis-SH3) domain, which then be able to bind proline-rich region (PRR) on p22phox and phox homology (PX) region that binds to phospholipids in the membrane. C-terminal PRR region on p47phox also allows binding of p47phox to SH3 domain on p67phox. RAC in its GDP form is post-translationally modified with geranyl-geranyl group, which is masked by RhoGDP-dissociation inhibitor (RhoGDI) to be maintained in the cytosol. Upon activation, GTP replaces GDP to dissociate RhoGDI, exposing the geanyl-geranyl group that allows RAC association with membrane lipids. This conformational change also releases binding site to NH2-terminal tetricopeptide repeat (TPR) on p67phox (Makni-Maalej et al. 2013, Lapouge et al. 2000). The last subunit, p40phox, interacts with p67phox through their phox and Bem1p (PB1) domain, to

participate in the complex assembly. NOX2 also subject to regulation of PI3K and phospholipase D, since they produce 3'-phosphorylated phosphatidylintositols and phosphatidic acid, respectively. Once formed, the complex transfer electron from NADPH from the cytosolic side to oxygen on the other side of the membrane, generating superoxide and subsequent production of other ROS.

NOX1 was the first homolog of gp91phox to be discovered (Banfi et al. 2000, Suh et al. 1999). It is most highly expressed in colon epithelium, as well as other tissues including vascular smooth muscle cells, endothelial cells, uterus etc. (Banfi et al. 2003, Suh et al. 1999, Szanto et al. 2005, Lassegue et al. 2001, Ago et al. 2005, Banfi et al. 2000). Superoxide genderation by NOX1 also depends on cytosolic subunits. NOX organizer 1 (NOXO1) is a homolog of p47phox with almost identical domain organization, except for absence of AIR domain. Similarily, NOX activator 1 (NOXA1) is homolog of p67phox, except lacking one SH3 domain spaning PB1 on p67phox. A study has suggested the non-specificity of NOX1 dependence on cytosolic subunit, in which NOX1 is able to produce superoxide when co-transfected with p47phox and p67phox (Banfi et al. 2003). However, NOX1 differs in the machnism of regulation by NOXO1 from that of gp91phox by p47phox. In contrast to translocalization of p47phox upon activation, NOXO1 constitutively locates on the membrane together with NOX1 and p22phox (Cheng and Lambeth 2004). This is because PX domain of NOXO1 binds to mono-phosphorylated phosphatidylinositols, which are abanduntly present in the resting membrane. Consequently, NOX1 shows weak constitutive activity when reconstituted in cells co-expressing NOXO1 and NOXA1. The full activation requires the PKC activator, suggested in a study using phorbol 12-myristate 13-acetate (PMA) (Geiszt et al. 2003).

In addition, plenty evidences have shown small GTPase RAC involves the regulation of NOX1 activity (Cheng et al. 2006). Therefore, the activity of NOX1 could be both constitutive and activable, depending on regulatory subunits that function analogously to NOX2.

NOX3 requires p22phox and NOXO1, and requirement for NOXA1 is specie-dependent (Ueno et al. 2005). The RAC dependence of NOX3 is still under debate, with two of the studies reporting independence, and another one suggesting its effect (Cheng et al. 2006, Ueno et al. 2005, Ueyama, Geiszt, and Leto 2006). NOX4 share the least homology with NOX2, and differ from other homologs with the way of activation. NOX4 is constitutively active, independent of subunits for further activation (Geiszt et al. 2000). Thus ROS production by NOX4 should be determined by its expression level or post-translational modification. The last class, including NOX5, DUOX1/2, posess calcium binding domain thus presumbaly activated by calcium.

3.2.2 Cellular Signaling

3.2.2.1 Inhibition of phosphatases

ROS regulates cellular signaling by multiple means. The most validated mechanism of ROS regulated signaling change occurs through its inhibion of redox-sensitive cysteine residues. For instance, protein tyrosine phosphatases (PTP) dephosphorylate their substrates, of which are numerous signal-transducing proteins. PTP contains cysteines in their catalytic region, which are susceptible to oxidation inactivation (Barford 2004). In another word, increased ROS production would inhibit PTP activity, thus enhance phosphorylation states in cells (Lee et al. 1998).

3.2.2.2 Activation of kinases

Hydrogen peroxide is known to activate p38 mitogen-activated protein (MAP) kinase through phosphorylation (Djordjevic et al. 2005). Various MAP kinases system elements have been proved subject to regulation of ROS, such as MAPK phosphatase-1 (MKP-1) , epidermal growth factor receptor (EGFR), c-Src, Ras, Akt (Griendling et al. 2000, Furst et al. 2005). However, it is not clear whether this activation is due to direct activation of upstream kinases, or the inactivation of PTP in previously stated section (Griendling et al. 2000).

3.2.2.3 Ca^{2+} signaling

NOX regulates intracellular calcium homeostasis through at least three components controling calcium flux: plasma membrane Ca^{2+} channel, intracellular Ca^{2+} channel and Ca^{2+} pumps. Thes channals are regulated by NOX through posttranslational modificaions, e.g. cysteine oxidation, S-glutathiolation, or electron transport-dependent cell depolarization.

Angiotensin II can increase the flux of L-type Ca^{2+} current, through angiotensin type 1 (AT1) receptor. This effect depends on NOX-derived ROS (Wang et al. 2004). In another study, it is shown that treatment of external hydrogen peroxide enhances neuronal voltage-dependent Ca^{2+} channel by accelerate the overall channel opening process (Li et al. 1998). However, the mechanism by which oxidation potentiates Ca^{2+} current still remains not entirely understood.

The second mechanism of calcium increase is from intracellular stores. Hydrogen peroxide treatment induces elevated intracellular Ca2+ concentration in the absence of extracellular calcium (Granados et al. 2006). In cardiomyoctyes, ischemic heart disease induced production of hydrogen peroxide may lead to contractile dysfunction, this could be attributed to protein kinase C-mediated sarcoplasmic reticulum calcium release (Wang et al. 1999). One

best characterized channel, ryanodine receptor family, contains reactive cysteine residues are subject to redox modification (Liu and Pessah 1994). Furthermore, this induction of increased calcium release is prevented by NOX blockers, apocynin or diphenyleneiodonium (DPI), but not other ROS-generating enzyme inhibitors (Cheranov and Jaggar 2006). Additional evidence suggests local NOX on the sarcoplasmic reticulum in coronary artery smooth muscle regulates ryanodine receptor activity by producing superoxide (Yi et al. 2006). On the other hand, the aforementioned study also suggested hydrogen peroxide only stimulates “Ca2+ sparks” and transient calcium activated potassium currents, leading to a global reduction of intracellular calcium (Cheranov and Jaggar 2006). This “Ca2+ spark” is likely due to activation of small cluster of ryanodine receptors.

The last compnent of calcium homeostatsis that is regulated by ROS is calcium pump (Adachi et al. 2004, Granados et al. 2006). ROS-related specie, nitric oxide-derived peroxynitrite (ONOO(-)), posttranslationally modifies sarco/endoplasmic reticulum calcium ATPase (SERCA) in the presence of glutathione at their cysteines residues, this S-glutathiolation increases SERCA’s calcium-uptake activity (Adachi et al. 2004). Finally, in human platelets, it is observed hydrogen peroxide induces Ca^{2+} release from agonist sensitive pool by modifiying SERCA and IP3 receptor’s sulphydryl group, this may contribute to explain enhanced platelet aggregation induced by hydrogen peroxide (Redondo et al. 2004).

3.2.3 NADPH oxidase inhibitor as a new antithrombotic strategy.

Altough ROS has been known to contribute thrombosis, antioxidants (e.g. Vitamins C, E and β-carotene etc.) do not appear to have the clinical efficacy to prevent cardiovascular events. (Morris and Carson 2003). Clinical trials weren’t be able to show any net benefit of antioxidant

intervention, in studies pertaining atherosclerosis or hypertension prevention (Bleys et al. 2006, Vivekananthan et al. 2003, Schiffrin 2010). Indeed, the lack of benefit in these clinical trials could be due to many reasons. First, none of them had actually reported if the administration of antioxidant had virtually affected the level of antioxidant concentration at the site of disease, for example, the vessle walls or thrombi. Second, even if they did, take Vitamine E for example, due to the extreme high rate constants of the reaction between ROS and endogenous molecules (like NO, amino acids, nucleic acids), which is often more than six orders of magnitudes higher than the constants between ROS and Vitamine E, the concentration of Vitamine E has to exceed that of NO about million folds to outcompete the other reaction. This is almost impossible to achieve by even the highest amount of Vitamine E administration. And lastly, even if the antioxidants had been able to react with ROS, the product could be simply another type of ROS that resistant to the original antioxidant. It is thus a more effective strategy to prevent ROS generation by targeting the enzymes producing them, than eliminate ROS once they are formed (Drummond et al. 2011).

Studies from both human and animal platelet has suggested the role of NADPH-dependent ROS in platelet activation and thrombosis. It has been reported, the presence of xanthine and xanthine oxidase system, which exogenously created superoxide, enhanced the sensitivity of platelet response to ADP. Moreover, when collagen or phorbol ester (PMA) were used as stimuli, O2- production was significantly increased. A peptide, gp91ds, specifically inhibiting the interaction between gp91phox (or its analogs) and p47phox prevented platelet O2- production, but not its scrambled analog as measured by chemiluminescence, or cytochrome c reduction (Krötz et al. 2002). Moreover, other evidences suggested ROS could modulate platelet

activation. Another study used increasing catalase, an enzyme that destroys H2O2, to show that during platelet activation, aggregation, ATP secretion, TXB2 release as well as calcium mobilization were all inhibited (Pignatelli et al. 1998).

Recent study from our lab demonstrated NOX1 and NOX2 differentially contribute to ROS production and regulates different platelet activation signaling pathways. And also, NOX2 is playing a critical role in thrombus formation *in vivo* as determined by laser-induced vascular injury model (Delaney et al. 2016).

Collectively, these evidence indicates NOX2 could be a potential target for antithrombotic strategy, as NOX are the major source of the ROS production during platelet activation, and NOX2 appears to be the major isoform involving this process.

II. PURPOSE OF STUDY

Data from our previous study answered the question of "what are the roles of individual NOX isoforms during platelet activation." We provided evidences to show that NOX1/NOX2-dependent ROS production facilitates platelet activation by promoting phosphorylation of Syk/PLCγ, and subsequent calcium mobilization (Delaney et al. 2016). Although it has been shown that NOX1 could be regulated through GPCR signaling pathway, while NOX2 could be regulated through both GPCR and GPVI/ITAM signaling pathway, the mechanism that involving NOX regulation upon agonist stimulation remains poorly understood (Fig. 3).

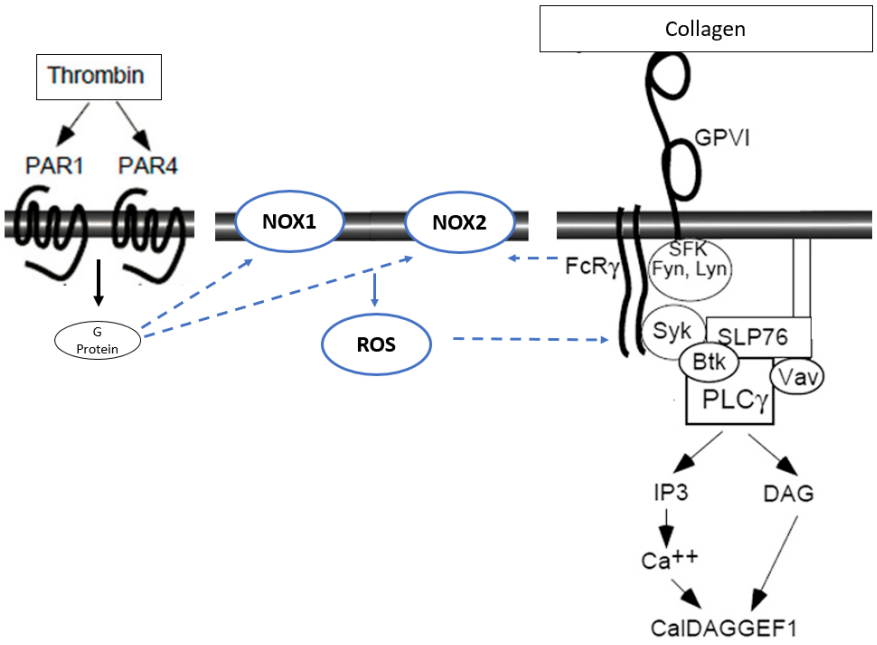


Figure 3. NOX1 and NOX2 are differentialy regulated in platelets. NOX1 could be regulated through GPCR signaling pathway, while NOX2 could be regulated through both GPCR and GPVI/ITAM signaling pathway. NOX1/NOX2-dependent ROS production facilitates platelet activation by promoting phosphorylation of Syk/PLCγ, and subsequent calcium mobilization. (Adapted from Li *et al*, 2010 with permission.)

One of the studies found that α2β1-integrin ligation could induce ROS production in Caco-2 cells (a colorectal adenocarcinoma cell line). By seeding cells on plates coated with various substrates or antibodies, they measured NOX-dependent ROS production induced by α2β1 integrin ligation. Collagen and Gi9 mAb are both high-affinity ligand of integrin α2β1, and they strongly induced NOX activity. Whereas laminin being a weaker agonist, it induced NOX activity to lesser extend (Honore et al. 2003).

Another report showed that compared with incubated in suspension, NIH-3T3 fibroblasts adhered on fibronectin, an integrin α5β1 ligand, exihibited dramatic increase in oxidants production. The redox state change subsequently resulted in inhibition of low molecular weight PTP (LMW-PTP), and secondary FAK acivation leading to cell adhesion and spreading onto fibronection (Chiarugi et al. 2003).

Thrombin receptor PAR1 and PAR4, like other GPCR, could activate integrin αIIbβ3 ligand binding function. Binding to extracellular ligands activates integrin outside-in signaling where SFK is required for the downstream events (Fig. 4). Based on the previous evidences, we hypothesized that thrombin-induced NOX2 activation is mediated through integrin αIIbβ3 signaling.

Therefore, the purpose of this study was to understand whether integrin αIIbβ3 was playing a regulatory role for NOX, especially NOX2, activity, and ROS production during platelet activation. We were also interested to know that if NOX-dependent ROS production was important for platelet fuctions, in specific, the integrin outside-in signaling and its funcitonal

consequences. By answering these questions, this study intended to provide a rationale for developing NOX(2) inhibitor as a new antithrombotic strategy.

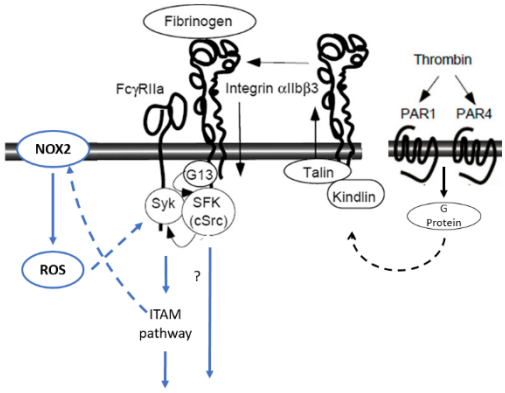


Figure 4. The schematic of hypothesized integrin-mediated NOX2 activation induced by thrombin stimulation. (Adapted from Li *et al*, 2010 with permission.)

III. METHODS AND MATERIALS

1 Mice

The generation of NOX1 and NOX2 knockout mice was described as before (Gavazzi et al. 2006, Pollock et al. 1995). Heterozygous female $NOX1^{+/-}$ mice were crossed with male C57BL/6 mice, which are purchased from Jackson Laboratory (Bar Harbor, ME). Male offspring $NOX1^{+/Y}$ were used as wild-type control (WT) and $NOX1^{-/Y}$ as NOX1 knockout (nox1 ko). NOX2 knockout mice (nox2 ko) were purchased from Jackson Laboratory (Bar Harbor, ME). Both strains were maintained on a C57BL/6 background. Integrin β3-/- mice were obtained from the Jackson Laboratory. Animal usage and protocol were approved by the institutional animal care committee of the University of Illinois at Chicago.

2 Materials

Src Family Kinase Inhibitor PP2, Syk inhibitor piceatannol, Akt inhibitors SH-6, PI3K inhibitors LY294002, PKC inhibitor Gö6979, p38 inhibitor SB203580, ERK1/2 inhibitor U0126, N-acetylcysteine were purchased from Calbiochem. Thrombin and fibrinogen were purchased from Enzyme Research Laboratories. dichlorodihydrofluorescein diacetate and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit, Alexa Fluor 546-conjugated phalloidin, were purchased from Invitrogen.

3 **Preparation of washed mouse platelets.**

Washed mouse platelets were prepared as previously described (Shen et al. 2013). Briefly, fresh blood was drawn from mouse inferior vena cava and anti-coagulated with ACD (85 mM trisodium citrate, 83 mM dextrose, and 21 mM citric acid). Platelets were isolated by differential centrifugation of whole blood with 0.1 μg/mL prostaglandin E1 and 1 U/mL apyrase (Sigma-Aldrich, St. Louis, MO). washed twice with CGS buffer (sodium chloride 0.12 M, trisodium citrate 0.0129 M, Dglucose 0.03 M, pH 6.5) and resuspended in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered Tyrode's solution containing 1 mM CaCl2 and 1 mM MgCl2. They were then allowed to rest at 25°C for at least 1 hour.

4 **Detection of intracellular ROS.**

Intracellular ROS was measured as previously described with modifications (Arthur et al. 2012). Briefly, washed platelets ($1x10^8$/mL) suspended in Tyrode's buffer were incubated with the global intracellular dye ROS dichlorodihydrofluorescein diacetate (H2DCFDA), or its close derivative, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H2DCFDA, Invitrogen) for 10-15 minutes. Agonist was added to platelet suspension at the start of test, and fluorescent signal was measured as a function of time using an Accuri C6 flow cytometer (BD,) to obtain kinetic changes in intracellular ROS. Total fluorescent intensity was determined by integrating the area under the curve (AUC). If shear stress was indicated, A cone and plate rheometer (Rheostress 1, Thermo-HAAKE, Paramus, NY) was used to introduce shear stress (800/s) to the platelets.

5 **Detection of hydrogen peroxide release.**

Washed platelets (3 X 10^8/ml) were stirred (1000 rpm) in a platelet aggregometer. Agonist was added to the tube and platelets were allowed to aggregate for 2 min. Reaction solution from Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) was added to the suspension and incubated for 30 min under 37 ºC before measured for fluorescent intensity using FlexStation plate reader (Molecular Devices).

6 **Immunoblotting.**

Washed platelets (3 X 10^8/ml) were pre-incubated with corresponding inhibitors or vehicle control, and stirred (1000 rpm) in a platelet aggregometer. Aggregation trace were recorded for 2 min after agonist was added to the tube, and reaction was stopped by addition of equal volume of 2 X SDS sample buffer (2% SDS, 0.1M Tris, 2% glycerol, 2 mM PMSF, 2 mM Na3VO4, 2 mM NaF, and Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Indianapolis, IN)). For platelets spreading on immobilized fibrinogen or incubated in suspension, they were lysed with the same 2 X sample buffer after indicated length of incubation. Proteins were separated by SDS-PAGE on 4-15% polyacrylamide gels, transferred to PVDF membranes and immunoblotted with the corresponding antibodies.

7 **Detection of ROS in platelets spread on immobilized fibrinogen.**

Microscope cover glasses (Fisher Scientific) were coated with 100 µg/mL fibrinogen (Enzyme Research Laboratories) in 0.1 M $NaHCO_3$ (pH 8.3) and blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Washed mouse platelets ($1x10^7$/mL) were allowed to adhere and spread on fibrinogen-coated wells (300 µL/well) at 37°C for indicated length of time. Washed mouse platelets ($1x10^7$/mL) were allowed to adhere and spread on fibrinogen-coated wells (300 µL/well) at 37°C for 60 min. 10µM H2DCF were then added to the suspension and platelets were incubated for another 30 min. Slides were aspirated to remove nonadherent platelets and washed with PBS for 6 times. ROS production was immediately observed using Zeiss LSM8 META confocal microscope. Quantification of the total ROS production per platelet was performed using NIH ImageJ software.

8 **Platelet spreading assay on immobilized fibrinogen.**

Microscope cover glasses (Fisher Scientific) were coated with 100 µg/mL fibrinogen (Enzyme Research Laboratories) in 0.1 M $NaHCO_3$ (pH 8.3) and blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Washed mouse platelets ($1x10^7$/mL) were allowed to adhere and spread on fibrinogen-coated wells (300 µL/well) at 37°C for indicated length of time. Slides were aspirated to remove nonadherent platelets and fixed with 4% paraformaldehyde, permeabilized, and stained with Alexa Fluor FITC conjugated phalloidin (Invitrogen). Adherent platelets were observed with a

Leica DM IRB fluorescence microscope (Leica Microsystems). Images were acquired using a Cool SNAP HQ CCD camera (Photometrics) and processed with NIH ImageJ software.

9 **Platelet adhesion under shear stress.**

Glass slides were coated with vWF (30 µg/Ml overnight. Slides were washed with PBS and blocked with 5% BSA in PBS for 1 hour and washed again with PBS. Washed mouse platelets (200µL of $3x10^8$/mL) were pretreated with either scramble DMSO or mP6 for 5 min, then loaded onto the slides. A cone and plate rheometer (Rheostress 1, Thermo-HAAKE, Paramus, NY) was used to introduce shear stress (800/s) to the platelets. Mepacrine (10 µM; Sigma), a fluourescent dye was added to the platelets before applying shear stress to the platelets for 5 minutes (Yin et al. 2008). Slides were rinsed in a container with 200 mL PBS for 3 times to wash out non-stably adherent platelets. Slides were viewed with a Leica 45 DMI RB fluorescence microscope (Leica Microsystems).

IV. RESULTS

1 Integrin αIIbβ3 is important for ROS production during platelet activation.

To determine if integrin αIIbβ3 is playing important role in regulating ROS generation during platelet activation, washed mouse platelets were treated with Integrilin, a reversible inhibitor that bind and inhibits integrin αIIbβ3, or citric acid as vehicle control. Platelets was stimulated with either thrombin or CRP to induce ROS production, which was measured by staining with florescent DCF using flow cytometry. Under static condition, ROS production was slightly reduced when treated with integrilin compared to control group (Fig. 5 A and B). However, when shear stress of 800/s was applied during stimulation, ROS production was significantly inhibited by integrilin (Fig. 5 C and D), in either thrombin- or CRP- induced platelet activation. This data indicates integrin αIIbβ3 is playing regulatory role in ROS production induced by thrombin or CRP stimulation, especially under shear stress (Fig. 6).

To validate this result, platelets isolated from either wild type (wt) or β3-/- mice were subject to the same assay. In consistence with the data before, mice platelets from β3-/- showed mild defects in ROS production under static condition, while were significantly defective in ROS production under shear stress, when stimulated with either low dose thrombin or CRP.

Since DCF is a dye that only staining intracellular ROS, we sought for another dye that could reflect the amount of ROS released from the cells. Amplex red reacts with extracellular H2O2 to produce the fluorescent oxidation product, resorufin. H2O2 is one of the major reactive oxidant species produced by platelets, and shown to be important for platelet

aggregation (Pignatelli et al. 1998). As determined by this assay, extracellular H2O2 generation by thrombin-stimulated platelets was also reduced in the presence of integrilin.

Collectively, these data suggest integrin is important in regulating ROS production during platelet activation, both intracellularly and extracellularly. Moreover, its contribution to ROS production is augmented under shear stress, which represents a more relevant setting since platelets are constantly subject to shear stress produced by blood flow under physiological condition.

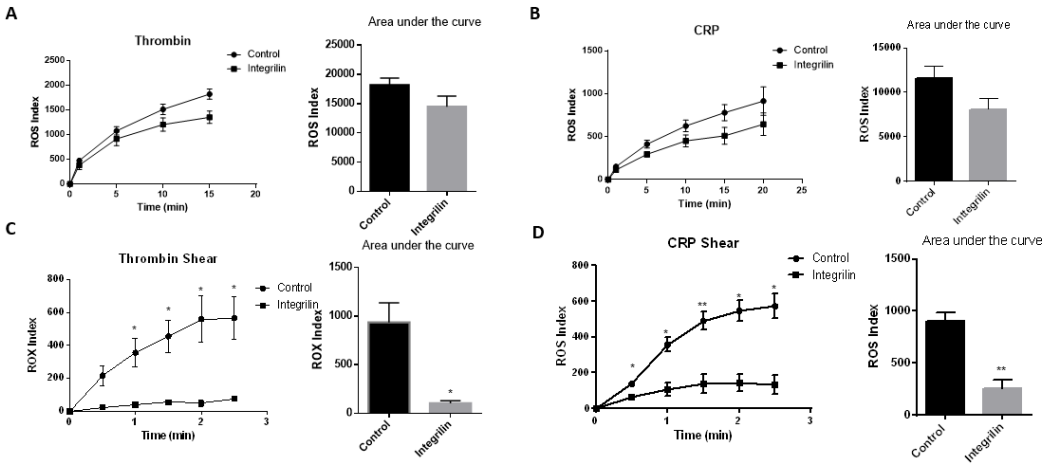
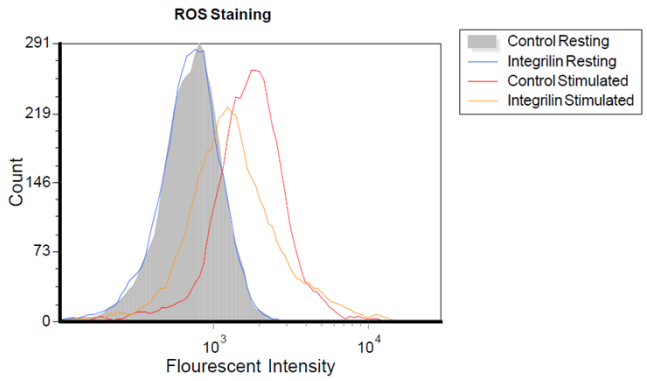


Figure 5. **ROS production in Integrilin-treated mice platelets.** (A-B) Isolated mouse platelets treated with vehicle control or integrilin (10µM) were incubated with carboxyl-H2DCFDA and stimulated with (A) 0.025U/mL thrombin or (B) 1µg/mL CRP. DCF fluorescence was measured at 1, 5, 10, 15 min using flow cytometry and quantified as mean ± SEM (n=8). (C-D) Isolated mouse platelets were incubated with carboxyl-H2DCFDA and stimulated with (C) 0.025U/mL thrombin or (D) 1µg/mL CRP under the shear rate of 800/s. DCF fluorescence was measured at 0.5, 1, 1.5, 2, 2.5 min using flow cytometry and quantified as mean ± SEM (n=3). (A-D) Total ROS production over time was calculated using area under the curve. (*P<0.05, **P<0.01, ***P<0.001, Student's t-test)

A



B

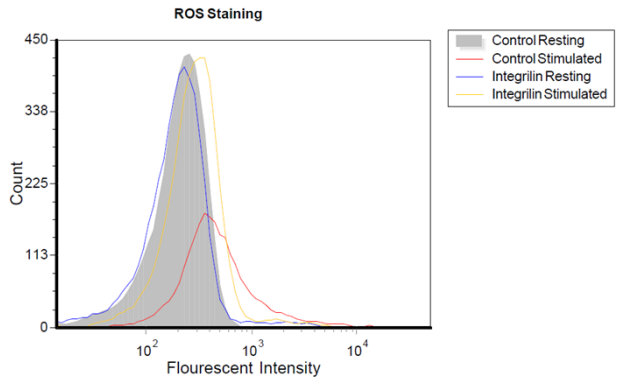


Figure 6. **Flow cytometric analysis of intracellular ROS production in platelets.** Platelets were activated with 0.025U/ml thrombin, treated with citric acid (control) or Integrilin (10µM) under resting state were used as negative control. Histogram shows relative fluorescence intensity on the x axis and number of events (count) on the y axis. (A) under static condition, platelets had been stimulated for 15 min. (B) under shear stress of 800/s, platelets had been stimulated for 2 min.

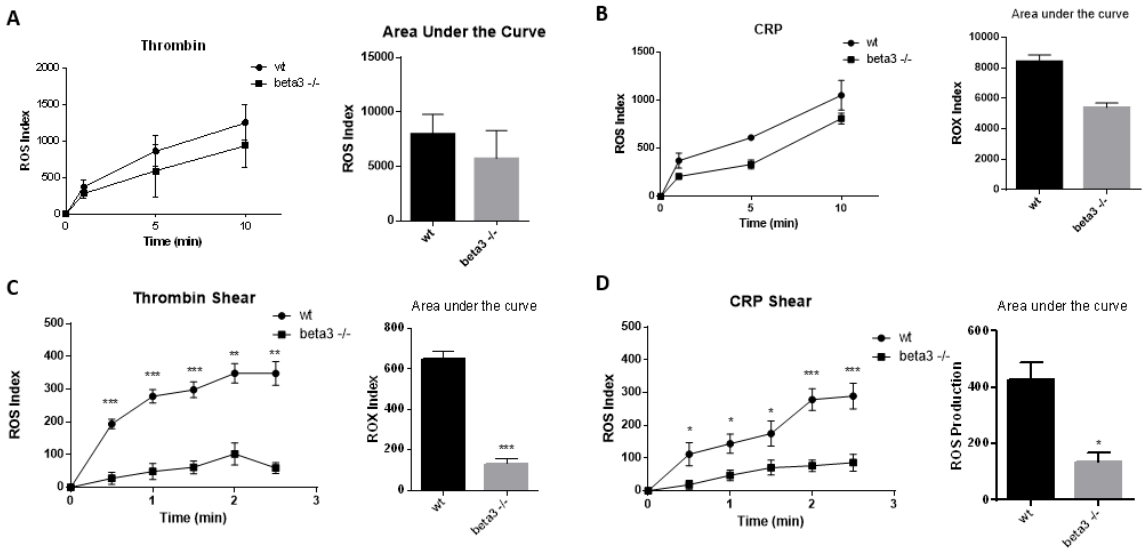


Figure 7. **ROS production in wt or beta3-/- mice platelets.** (A-B) Isolated platelets from wt or beta3-/- mice were incubated with carboxyl-H2DCFDA and stimulated with (A) 0.025U/mL thrombin or (B) 1µg/mL CRP. DCF fluorescence was measured at 1, 5, 10 min using flow cytometry and quantified as mean ± SEM (n=3). (C-D) Isolated platelets from wt or beta3-/- mice were incubated with carboxyl-H2DCFDA and stimulated with (C) 0.025U/mL thrombin or (D) 1µg/mL CRP under the shear rate of 800/s. DCF fluorescence was measured at 0.5, 1, 1.5, 2, 2.5 min using flow cytometry and quantified as mean ± SEM (n=4, 7). Total ROS production over time was calculated as area under the curve. (*P<0.05, **P<0.01, ***P<0.001, Student's *t*-test)

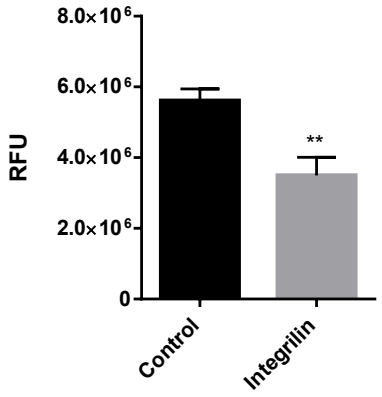


Figure 8. **Hydrogen Peroxide release from activated platelets.** Staining hydrogen peroxide released during platelet activation using Amplex Red. Platelets was stimulated with 0.025U/ml thrombin, treated with either vehicle control or integirlin (10µM). Platelet aggregation was induced using a turbidometric aggregometer at 37°C and 1000 rpm stirring speed. Amplex red is added after 5min aggregation and allowed to incubate for 30min. RFU was measured using fluorescent plate reader. (n=6, **P<0.01, Student's t-test.)

2 **Integrin-dependent ROS production is stimulated by agonist and/or shear stress.**

To determine what is the stimulus that induces this integrin-dependent ROS production, we applied either shear stress or agonists alone, and measured the ROS production under different conditions. Compared with resting state, either shear stress or agonist alone (low dose thrombin or CRP) was be able to induce ROS production to a certain level, but neither as high as combined (Fig. 9). Therefore, we identified thrombin/CRP and shear stress both as stimuli capable of inducing ROS production in platelets.

Under constant shear stress, increasing concentration of thrombin induced dose-responsive ROS production in platelets (Fig. 10). While in the other group where platelets were treated with integrilin, ROS production was constantly inhibited, regardless of the dose of thrombin. Result indicates thrombin-stimulated ROS production is integrin dependent.

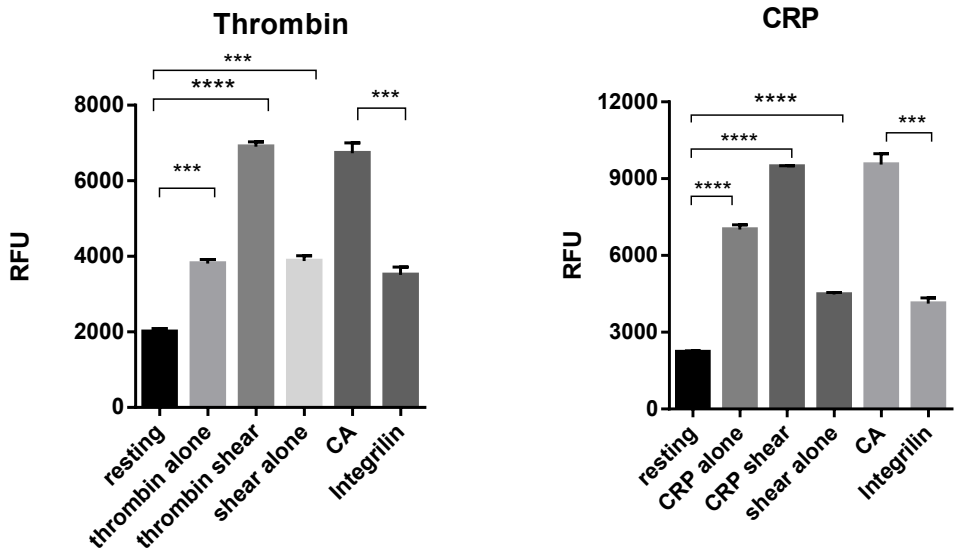


Figure 9. **Stimuli that induce platelet ROS production.** Isolated platelets from wild type mice was stimulated with agonists (0.025U/ml thrombin or 1 µg/ml CRP), shear stress alone, or combined. After 2min of stimulation, platelets are stained with 10µM H2DCFDA and florescence is measured with flow cytometry, quantified as mean ± SEM (n=3). (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, Student's *t*-test)

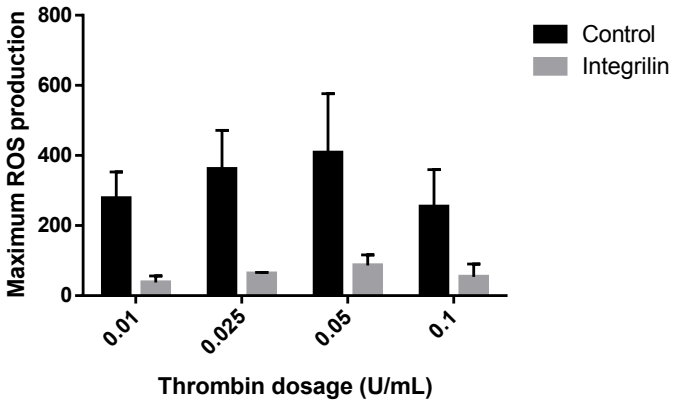


Figure 10. **ROS production in platelets are thrombin dose-responsive.** Isolated mouse platelets treated with vehicle control or integrilin (10µM) were stimulated with 0.01, 0.025, 0.05, 0.1U/mL thrombin under shear stress for 2min. Platelets are stained with 10µM carboxyl-H2DCFDA and florescence is measured with flow cytometry, quantified as mean ± SEM (n=2).

3 **Activation of p47phosphorylation is integrin αIIbβ3-dependent**

To evaluate the activity of NOX2, which was proven to be the major isoform involved in ROS production leading to platelet activation and thrombosis, phosphorylation of p47phox at Ser304 was used as an indicator. Signaling triggers such as protein kinase C and AKT phosphorylate p47phox, the organizer subunit, at its autoinhibitory region (AIR). Serine residues in this region become extensively phosphorylated, up to as many as nine sites. Among them, Ser303/304 are two residues that are of special interest because alanine double mutant of Ser303/304 almost completely abolished NOX activity (Inanami et al. 1998, Hoyal et al. 2003).

Platelet aggregation induced by thrombin or CRP was strongly inhibited by integrilin, which also inhibited ROS production in platelet measured after aggregation (Fig. 11A). Compared with resting state, p47phox was phosphorylated when platelets were stimulated by thrombin or CRP, but showed marked reduction in β3-/- or integrilin treated platelets (Fig. 11B). In wt mice platelets, p47phox phosphorylation was enhanced with increasing dose of thrombin, while it was strongly reduced in β3-/- platelets. When CRP was used as agonist, there was only partial reduction in β3-/- platelets, indicating possible integrin-independent pathway for p47phox activation (Fig. 11C).

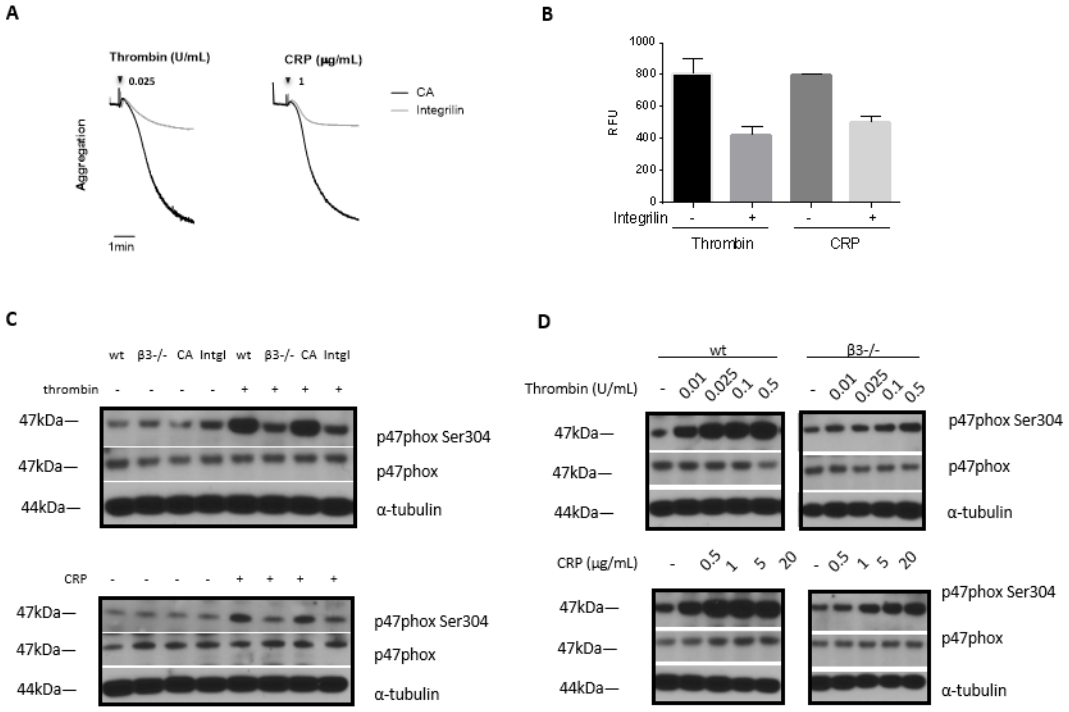


Figure 11. **ROS production and p47phox phosphorylation are integrin-dependent.** (A) representative aggregation trace of thrombin (0.025U/ml) or CRP (1μL/ml) –stimulated platelets, in the presence of vehicle control (CA) or Integrilin (10μM). Platelets are diluted and stained with H2DCFDA after 2min aggregation. (B) ROS production is determined by RFU with flow cytometry. (C) platelets are lysed under resting condition or after 2min aggregation induced by thrombin (0.025U/ml) or CRP (1μL/ml), probed for p47phox Ser304, total p47phox or α-tubulin. (D) wt and β3-/- mice platelets are lysed under resting condition or after 2min aggregation induced by increasing dose of thrombin (0.01, 0.025, 0.1, 0.5U/ml) or CRP (0.5, 1, 5, 20μL/ml), then probed for p47phox Ser304, total p47phox or α-tubulin.

4 **Thrombin-induced p47phox activation requires SFK but not Syk.**

To identify the pathway responsible for p47phox activation, downstream kinases involved in integrin-dependent signaling were tested to determine their roles in thrombin-induced p47phox phosphorylation. Src family kinases (SFKs) are a group of closely related nonreceptor protein tyrosine kinases. c-Src, for example, is shown to constitutively bind to integrin β3 through an interaction involving the c-Src SH3 domain and β3 cytoplasmic tail (Arias-Salgado et al. 2003). They are playing essential role in platelet integrin outside-in signal, and consequential functions such as stable adhesion, spreading, secretion etc (de Virgilio, Kiosses, and Shattil 2004, Arias-Salgado et al. 2005, Li, Zhang, et al. 2010). In addition, previous studies demonstrated fibrinogen binding to integrin induces rapid Src activation which promotes Syk association to integrin-c-Src complex (Gao et al. 1997, Obergfell et al. 2002).

By preincubating platelet with SFK inhibitor PP2, p47phox phosphorylation induced by thrombin stimulation was markedly reduced (Fig. 12A), suggesting p47phox activation is downstream of integrin outside-in signaling pathway. However, Syk inhibitor, piceatannol, was not be able to change the phosphorylation level of p47phox (Fig. 12B). Collectively, we conclude that p47phox activation in thrombin induced by thrombin is downstream of SFK-dependent integrin outside-in signaling, but does not require Syk activity.

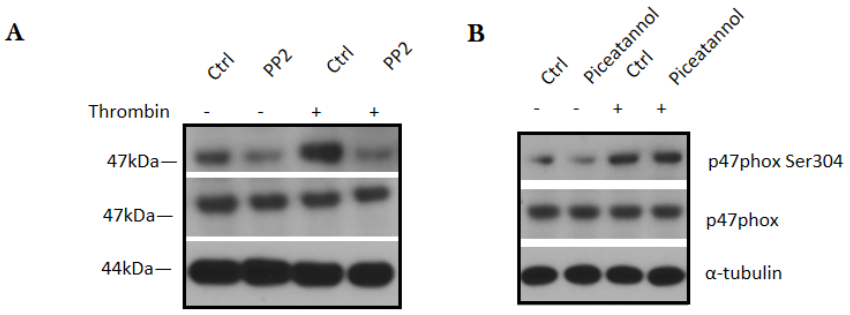


Figure 12. **Thrombin-induced p47phox activation requires SFK but not Syk.** Isolated platelets are preincubated with (A) PP2 (10µM), (B) piceatannol (15 µM) or DMSO as control and then lysed under resting condition or after 2min aggregation induced by thrombin (0.025U/ml), probed for p47phox Ser304, total p47phox or α-tubulin.

5 CRP-induced p47phox activation requires both SFK and Syk.

SFK family kinases, Lyn and Fyn, are constitutively associated with GPVI-FcRγ complex (Suzuki-Inoue et al. 2002), and are required to phosphorylated tyrosines with ITAM domain of FcRγ, leading to activation of the tyrosine kinase Syk and a series of downstream events (Ezumi et al. 1998).

When stimulated with GPVI agonist CRP, p47phox phosphorylation was also induced while inhibited when platelets are pretreated with PP2 or piceatannol. Hence, unlike integrin outside-in signaling, p47phox activation by GPVI/ITAM pathway requires both SFK and Syk.

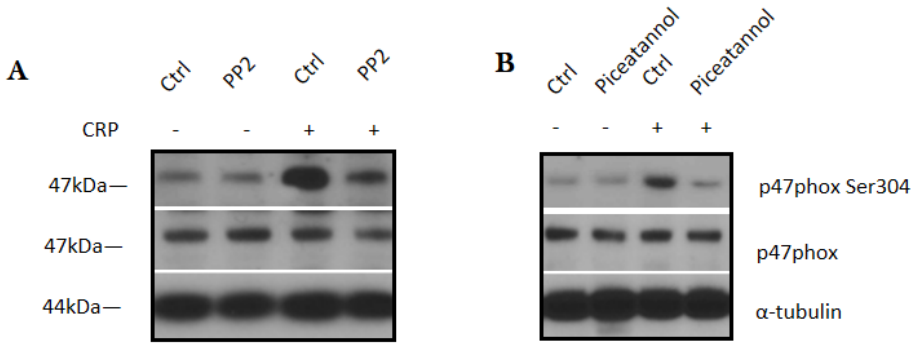


Figure 13. **CRP-induced p47phox activation requires both SFK and Syk.** Isolated platelets are preincubated with (A) PP2 (10μM), (B) piceatannol (15 μM) or DMSO as control and then lysed under resting condition or after 2min aggregation induced by CRP (1μg/ml), probed for p47phox Ser304, total p47phox or α-tubulin.

6 **Activation of p47phox depends on PI3K/Akt activity.**

Since both SFK and Syk are tyrosine kinases, we investigated further to determine the serine/threonine kinases downstream that are responsible for activation of p47phox. In neutrophils, activation of appropriate receptors activates PKC and PI3K leading to p47phox phosphorylation (Ding et al. 1995, Vlahos et al. 1995, Didichenko et al. 1996, Fontayne et al. 2002). It has also been suggested that activation by PI3K of p47phox is carried out by Akt activity, since Akt is able to phosphorylate p47phox on Ser304 and Ser328 in cell free settings (Hoyal et al. 2003). Some studies also suggest involvement of MAPKs in activating p47phox (El Benna et al. 1996).

In thrombin-stimulated platelets, phosphorylation of p47phoxSer304 was partially inhibited by PI3K inhibitor, LY294002, and strongly inhibited by Akt inhibitor, SH-6 (Fig. 14A), while was not changed in the presence of MAPK or PKC inhibitors (Fig. 14B). On the other hand, phosphorylation of p47phoxSer304 in CRP-stimulated platelets was also reduced by Akt and PI3K inhibition, but were completely blocked by PKC inhibitor, Gö6979, as well (Fig. 14C, D).

These data suggest PI3K/Akt signaling is important for p47phox activation regardless of the activation pathways. On contrary, PKC is only important for CRP-induced p47phox activation, while we do not exclude the possibility that PKC contributes to p47phox activation by phosphorylating other serine sites.

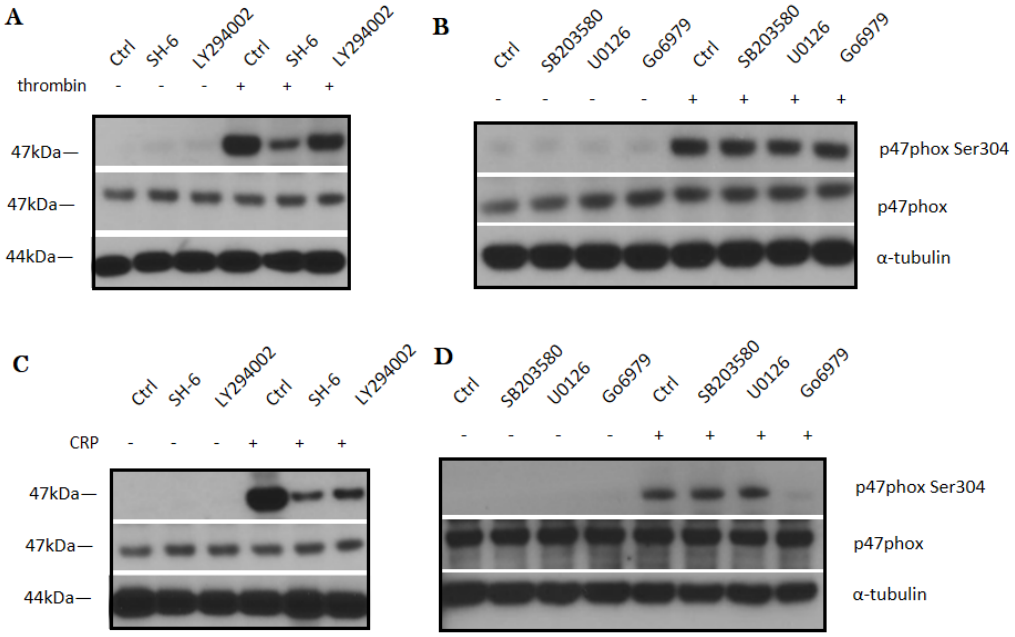


Figure 14. **PI3K/Akt is important for p47phox phosphorylation on Ser304.** Platelets are pretreated with (A, C) Akt inhibitor, SH-6 (15µM), or PI3K inhibitor, LY294002(5µM) (B, D) p38 inhibitor, SB203580 (10µM), ERK1/2 inhibitor, U0126 (250nM) or PKC inhibitor, Gö6979 (50nM), then stimulated with (A, B) thrombin (0.025U/ml) or (C, D) CRP (1µg/ml) to induce aggregation. Cells are lysed under resting condition or after 2min aggregation, probed for p47phox Ser304, total p47phox or α-tubulin. DMSO was added as control.

7 **Integrin-ligation is sufficient to induce ROS production in platelets.**

Platelet spreading is a signature event mediated by integrin αIIbβ3 outside-in signaling (Li, Delaney, et al. 2010). After talin binds to β3 to mediate inside-out signaling, it is dissociated from β3 stimulated by integrin-ligand binding. This dissociation allows Gα13 bind to an ExE motif within the β3 tail (Shen et al. 2013). Gα13-β3 interaction induces outside-in signaling through the phosphorylation and activation of the c-Src (Gong et al. 2010). In the early phase of outside-in signaling, c-Src activates RhoGAPs (Rho GTPase Activating Proteins), such as p190RhoGAP, promoting RhoA-GTP converting to RhoA-GDP (Arthur and Burridge 2001).Therefore, Src inhibits the RhoA-dependent retractile signaling pathway, leading to cell spreading.

mP6 is a myristoylated peptide that inhibited interaction of αIIbβ3 with Gα13, thus diminished activation of protein kinase c-Src and outside-in signaling (Shen et al. 2013). mP6 inhibited platelet spreading on fibrinogen, but had no effect on either agonist-induced fibrinogen or PAC, which is a ligand-mimetic antibody recognizing activated αIIbβ3, binding to platelets, or platelet adhesion to immobilized fibrinogen. Data proved that mP6 selectively inhibits the early phase of outside-in signaling without affecting talin-dependent inside-out signaling, and ligand-induced integrin activation.

Consistent with previous data, in the presence of thrombin, ROS production was significantly raised compared to platelets adhered to poly-l-lysine coated plates, when spreading on fibrinogen, while strongly inhibited by mP6 but not the scramble peptide (Fig. 15). More interestingly, spreading on fibrinogen alone was enough to give rise to ROS production,

which was also inhibited by mP6. This data strongly support that ROS production is regulated by integrin αIIbβ3, mainly through the outside-in signaling. Moreover, it suggests integrin ligation and outside-in signal is sufficient to induce ROS production, without need for other type of stimulation.

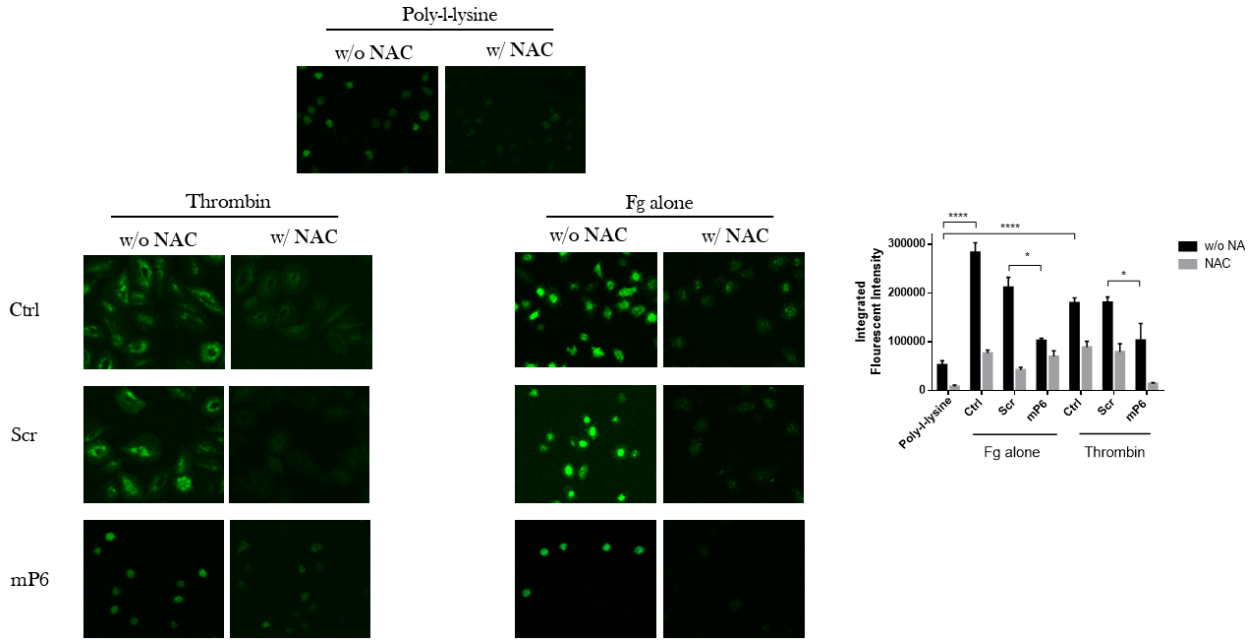


Figure 15. **ROS production in spreading platelets.** Mouse platelets were allowed to spread on fibrinogen-coated slices for 90 min, incubated under 37ºC, with or without the presence of 0.01U/ml thrombin. Platelets treated with N-acetylcysteine (NAC) were used as negative control. 10μM DCF were added during the last 30min of incubation. Platelets were then washed and observed under the fluorescent microscope (left). Total ROS production per cell were calculated by integrating the fluorescent intensity (right). (*P<0.05, Student's t-test, n=75,100,2,95,45,9,19,6,40,47,6,10.)

8 **Integrin-ligation is able to induce p47phox activation.**

Then to test whether integrin signaling is required for NOX2 activation, isolated mouse platelets were allowed to spread on slides coated with fibrinogen, treated with fibrinogen alone, MnCl2 or CRP. MnCl2 is a direct activator of integrin outside-in signaling, bypassing any need for inside-out signaling (Zhang et al. 2011). As shown, platelets treated with MnCl2 and CRP spread better than on fibrinogen alone (Fig. 16A). Meantime, western blot for p47phox revealed slightly increased phosphorylation of p47phox in platelets spread on fibrinogen alone, and strong enhancement in MnCls treated platelets (Fig. 16B, C). In the latter case, p47 phosphorylation was induced as quick as 5mins, and significantly higher than resting cells at 30min. This validated our conclusion that NOX2 is downstream of integrin outside-in signaling pathway. At the same time, addition of CRP instead of MnCl2 as agonist induced much higher activation at beginning. This indicates that there might be additional GPVI-mediated signaling that also activates NOX2, which is mainly responsible for NOX2 early phase activation.

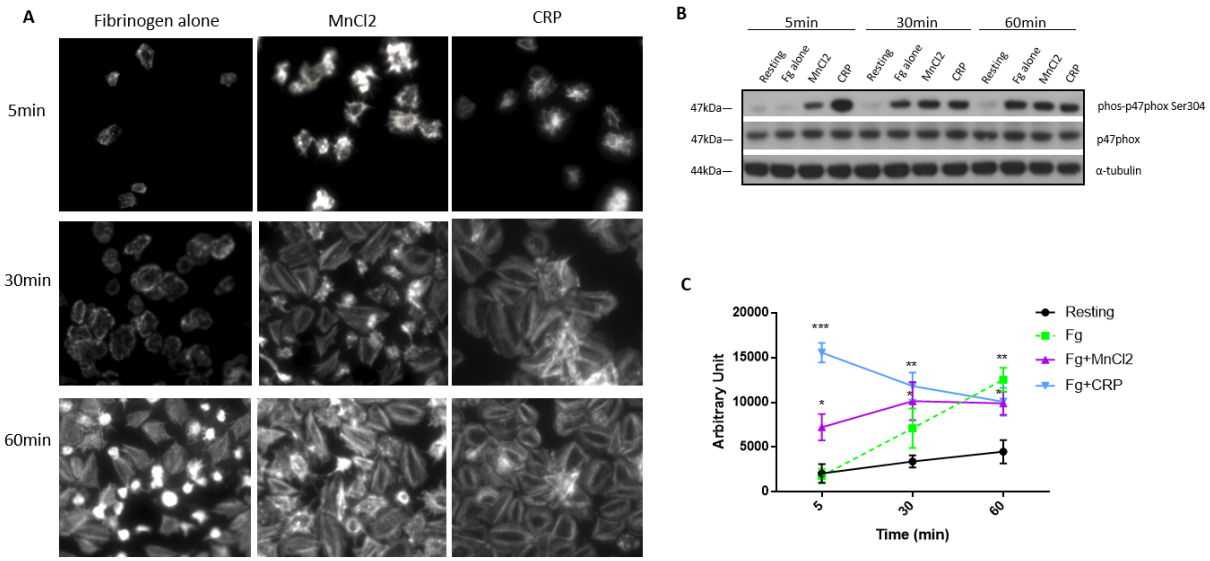


Figure 16. **Integrin-ligation activates p47phox during platelet spreading.** (A) Mouse platelets were allowed to spread on fibrinogen-coated slices for indicated length of time, incubated under 37ºC. In other groups, platelets were also treated with 0.5mM MnCl2 or 1µg/ml CRP. Adherent platelets were fixed, permeabilized, and stained with phalloidin and observed with fluorescence microscope. (B) Adherent platelets were collected and lysed, probed for p47phox Ser304, total p47phox or α-tubulin. Platelet kept in suspension were used as resting control. (C) quantification of western blot signal intensity for p47phox. Normalized by the average of total p47phox and α-tubulin. (*P<0.05, **P<0.01 ***P<0.001, Student's t-test, n=4.)

To confirm the involvement of β3, wt mouse platelets was compared with beta3-/-, treated with either resting condition, fibrinogen alone, MnCl2, or CRP. After 30mins incubation, cells were collected and lysed. Western blot showed NOX2 activation was equally abolished across groups (Fig. 17). Based on above results, we conclude that although nox2 can alternatively be activated through GPVI-mediated pathway, this activation is taken place only at early phase.

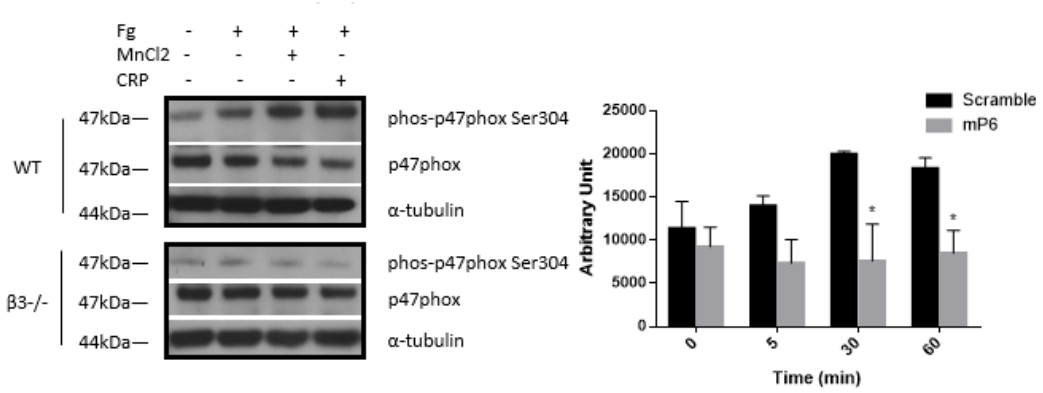


Figure 17. Fibrinogen-ligation induced p47phox phosphorylation depends on β3. (A) Isolated wt or β3-/- mouse platelets were incubated under resting states, or with either fibrinogen alone (250μg/ml), MnCl2 (0.5mM/ml), or CRP (1μg/ml), as indicated, for 30 min under 37°C. Platelets were then lysed and blotted for respective proteins. (B) Quantification of western blot signal for p47phox Ser304 phosphorylation, normalized by average signal of total p47phox and α-tubulin. Plotted as mean ± SEM (n=4). (*P<0.05, **P<0.01, ***P<0.001, ns, non-significant, Student's *t*-test)

9 **Platelet spreading induced phosphorylation of p47phox is inhibited by mP6.**

To confirm the involvement of integrin outside-in signal in NOX2 activation, platelets were seeded on fibrinogen treated with either scramble peptide or mP6. Platelets in the mp6-treated group were defective in spreading, round-shaped compared to control group (Fig. 18A, B). This validated the effectiveness of mp6 in blocking outside-in signaling, since spreading is a key event induced by integrin activation. Collected platelet was lysed and blotted for phospho-p47phox. Compared to scramble peptide, mp6 prevented p47phox phosphorylation as well (Fig. 18C, D). These data verified our conclusion that p47phox activity could be activated through integrin outside-in signal.

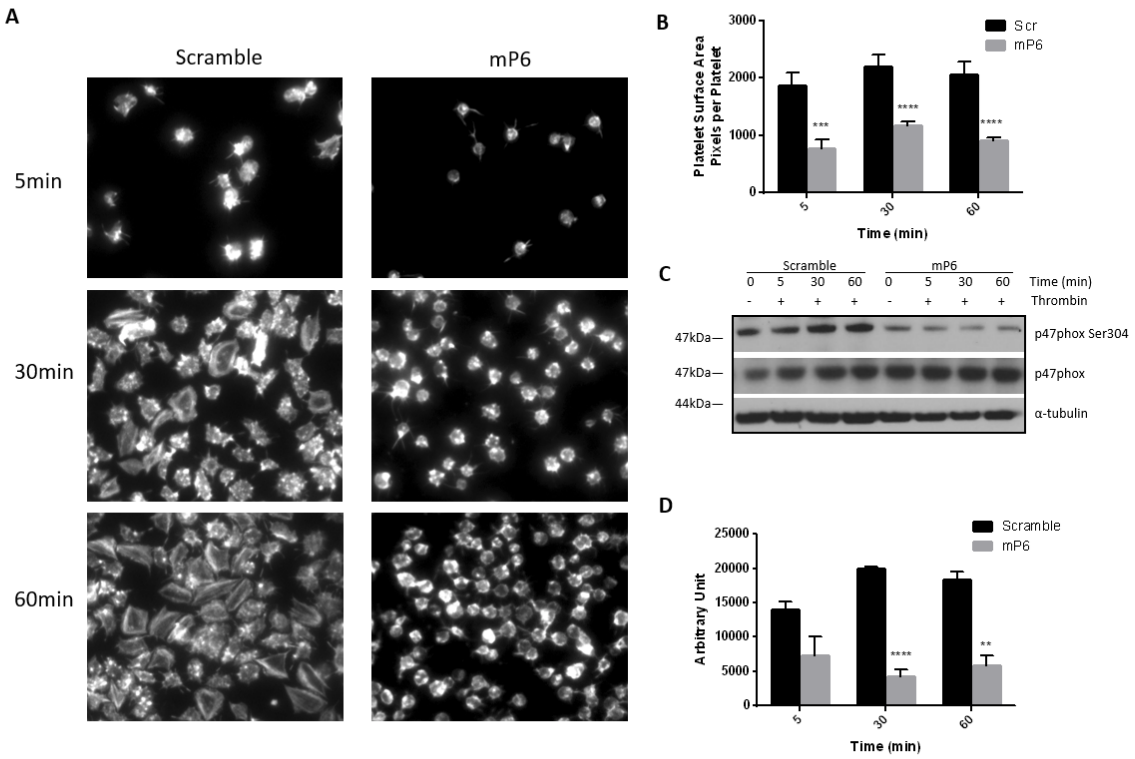


Figure 18. **Platelet spreading and spreading induced phosphorylation of p47phox are inhibited by mP6.** (A) Isolated mouse platelets were allowed to spread on slides coated with 100µg/mL fibrinogen for 5, 30, 60 min at 37°C. Platelets were incubated with 100µM scramble peptide or mP6 before loading on to the slides. Adherent platelets were fixed, permeabilized, stained with phalloidin, and then observed with a fluorescence microscope. (B) Surface area of single platelets was quantified using ImageJ and plotted as mean ± SEM (n=20). (C) spread plated were collected then lysed for immunoblotting. Resting platelets were used as negative control. (D) Quantification of western blot signal for p47phox Ser304 phosphorylation, normalized with average intensity of total p47phox and α-tubulin. Plotted as mean ± SEM (n=3). (***P<0.001, ****P<0.0001, Student's *t*-test)

10 **NOX1 and NOX2 are important ROS generator in platelets.**

Data above showed evidences on platelets ROS production could be induced by αIIbβ3 activation, and it depends on integrin outside-in signaling. It was also shown that NOX2 activation could be induced by ligand binding of integrin αIIbβ3, which depends on integrin outside-in signaling as well. But we still need to determine whether or not under this setting, NOX plays an important role in overall platelet ROS production.

To answer this question, wt, nox1 ko, or nox2 ko mice platelets were activated with low dose thrombin under either static status or shear stress, as measured by flow cytometry (Fig. 19). And then, overall ROS production of nox1 ko and nox2 ko platelets after they were induced to spread on fibrinogen-coated plates were measured. Compared to wt, nox1 ko and nox2 ko both showed significant defect with aspect to total ROS production (Fig. 20). These data therefore confirmed NOX1 and NOX2 are major generators of platelet ROS production induced by integrin activation.

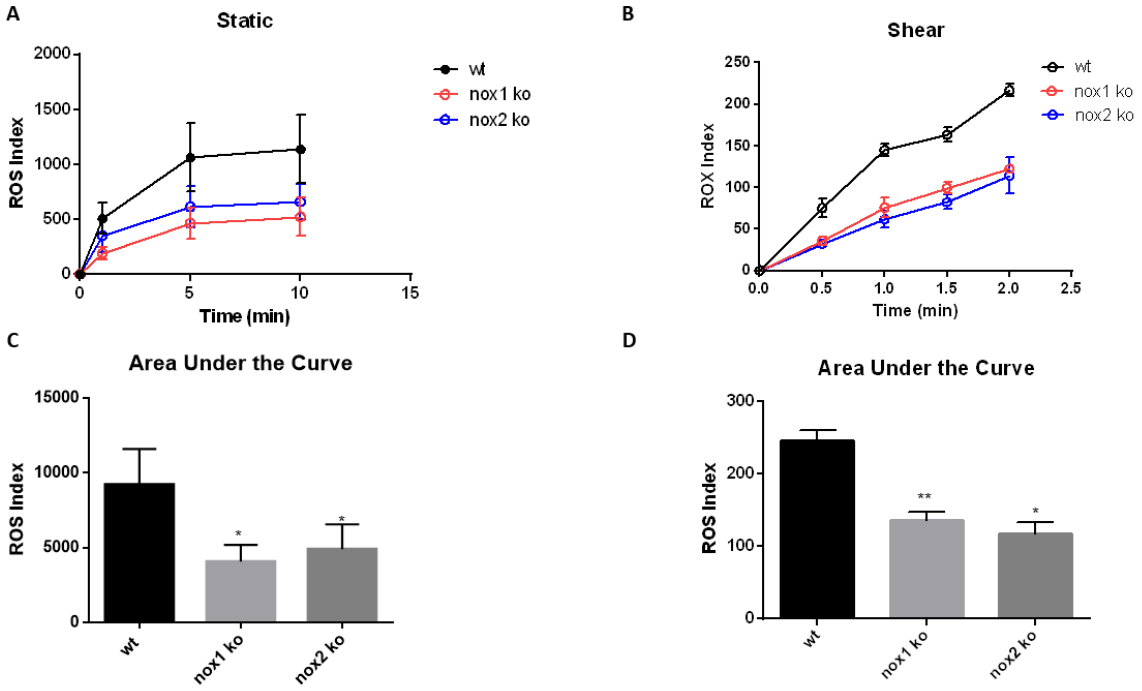


Figure 19. **ROS production in NOX knockout mice platelets.** (A-B) wt, nox1 ko, or nox2 ko mice platelets was incubated with carboxyl-H2DCFDA and stimulated with 0.025U/mL thrombin. (A) Stimulation was under static condition and DCF fluorescence was measured at 1, 5, 10, 15 min using flow cytometry and quantified as mean ± SEM (n=6). (B) Stimulation was under shear rate of 800/s and DCF fluorescence was measured at 0.5, 1, 1.5, 2, 2.5 min using flow cytometry and quantified as mean ± SEM (n=4). (C)Quantification of ROS production under static condition over time as area under the curve. (D) Quantification of ROS production under shear condition over time as area under the curve. (*P<0.05, **P<0.01, ***P<0.001, Student's *t*-test)

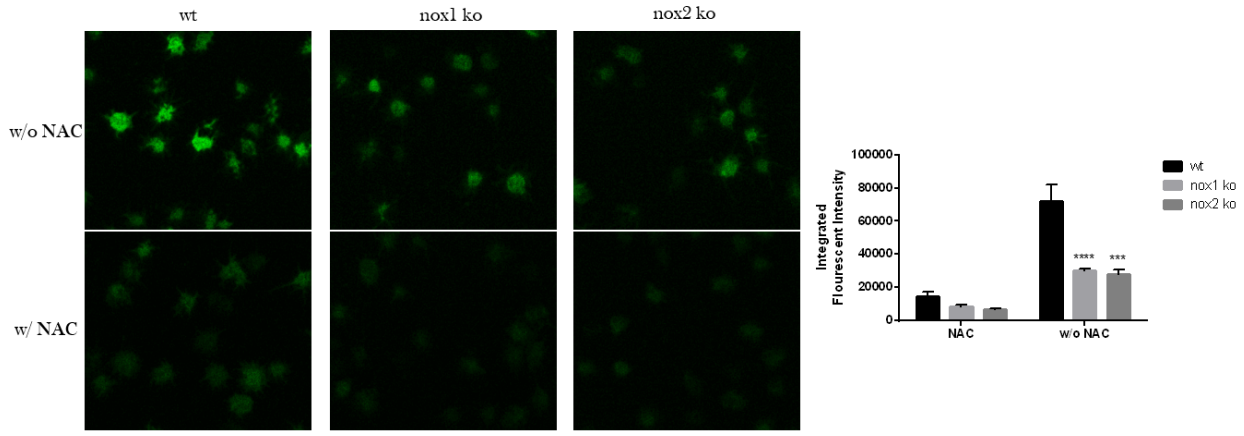


Figure 20. **ROS production in spreading platelets of NOX knockout mice.** Mouse platelets were allowed to spread on fibrinogen-coated slices for 90 min, incubated under 37ºC, with or without the presence of 0.01U/ml thrombin. Platelets treated with N-acetylcysteine (NAC) were used as negative control. 10µM DCF were added during the last 30min of incubation. Platelets were then washed and observed under the fluorescent microscope (left). Total ROS production per cell were calculated by integrating the fluorescent intensity (right). (*P<0.05, Student's t-test, n=75,100,2,95,45,9,19,6,40,47,6,10.)

11 Integrin –dependent stable adhesion is impaired in NOX knockout platelets.

To investigate whether NOX1 and NOX2 affect platelet outside-in signaling and its functional consequences, we first tested the influence of NOX on platelet stable adhesion. While mP6 does not inhibit resting platelet adhesion to immobilized fibrinogen (Shen et al. 2013), it reduced the number of adherent platelets on immobilized VWF significantly. NOX1 and NOX2 knockout platelets were comparably defective in stable adhesion, which was not further inhibited by mp6 (Fig. 21). This could possibly suggest ROS reversely inhibits integrin outside-in signaling.

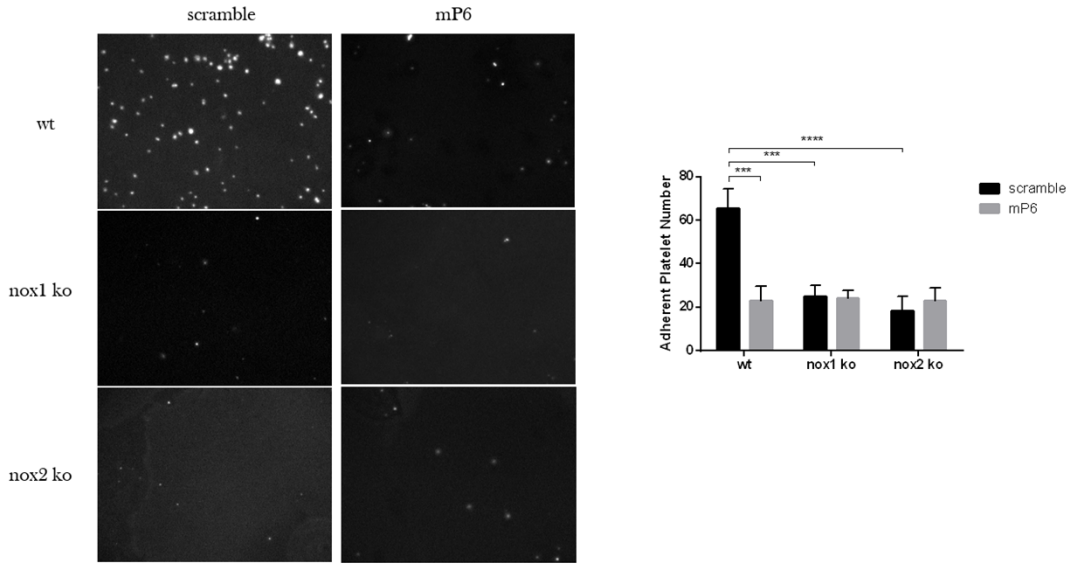


Figure 21. Integrin-dependent stable platelet adhesion of NOX knockout platelets. Left: Isolated platelets of wt, nox1-/Y, or nox2-/- mice were pre-incubated with DMSO or mP6, loaded onto slides coated with 30µg/mL vWF, adhesion was induced by applying 800/s shear stress for 5min. Mepacrine was added to the platelets for fluorescent staining before applying shear stress. Slides were viewed with a fluorescence microscope. Right: quantitation of stably adhered platelets in 30 random fields, using ImageJ software. Adherent platelet number is quantified as mean ± SEM. (*P<0.05, **P<0.01, ****P<0.0001, Student's *t*-test)

12 **Platelet spreading is not affected by NOX knockouts.**

Next, to determine if NOX1 and NOX2 are important for platelet spreading, wt, nox1 ko or nox2 ko platelets spread on fibrinogen-coated plate were measured with surface area. The result showed there was no significant difference between the wt and nox knockout platelets (Fig. 22). In addition, when stimulated with low dose thrombin, nox knockout platelets didn't differ from wt either (data not shown).

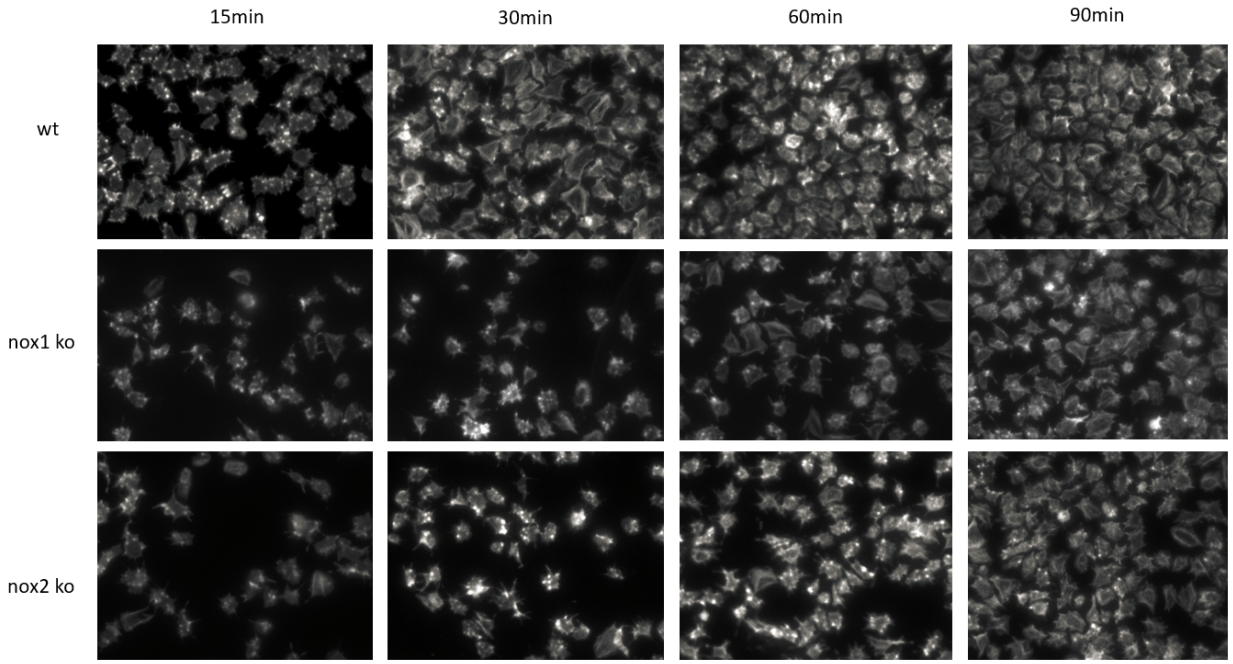


Figure 22. **Platelet spreading of NOX knockout platelets.** Isolated platelets of wt, nox1-/Y, or nox2-/- mice were allowed to spread on slides coated with 100µg/mL fibrinogen for 15, 30, 60, 90 min at 37°C. Adherent platelets were fixed, permeabilized, stained with phalloidin, and then observed with a fluorescence microscope.

13 Discussion

Although ROS has been known to contribute to thrombosis, antioxidants (e.g. Vitamins C, E and β-carotene etc.) do not appear to have the clinical efficacy to prevent cardiovascular events (Morris and Carson 2003). This is due to poor reactivity of conventional antioxidant with many endogenous ROS, low bioavailability at the site of disease, and many other limitations (Drummond et al. 2011). Therefore, preventing ROS formation by targeting the enzymes responsible for their generation could be a more effective strategy for ameliorating oxidative stress than scavenging ROS once they are formed. The data shown here have provided evidence of NOX being the major resources of ROS during platelet activation and hence the most logical therapeutic targets.

Next, data have indicated NOX2 would be a superior target for drug intervention than other isoforms because it is the major member involves in platelet activation and thrombosis, despite of the type of stimuli. *In vivo* data in mice also supported this by showing NOX2 is important for thrombus formation but not hemostasis process. (Delaney et al. 2016). To achieve this, one possibility is to target at p47phox instead of the catalytic NOX subunits, which are quite similar between isoforms. P47phox is the only subunit that is used specifically by NOX2 oxidase, and by the NOX1 oxidase expressed in vascular smooth muscle cells (Ambasta et al. 2006). Since NOX1 in VSMC is also a proved contributor for cardiovascular diseases, targeting p47phox would provide reasonable selectivity over other isoforms and cell types, thus being a superior strategy for preventing cardiovascular diseases.

This study has also provided rationale for developing NOX inhibitor that targeting its upstream integrin outside-in signaling pathway as new anti-thrombotic strategy. It has been shown that integrin αIIbβ3 is important for ROS production in activated platelets under both static condition or shear stress. As platelets are constantly subject to shear stress produced by blood flow under physiological conditions, it is intriguing to find augmented contribution of integrin to ROS production in this setting. We also identified thrombin/CRP and shear stress both as stimulus to induce ROS production in platelets. Since later dose-response test reveals thrombin-induced ROS production was completely blocked by integrilin, it is suggested thrombin-induced ROS production is integrin-dependent. To illustrate by what means does integrin regulate ROS production, p47phox phosphorylation was detected as an indicator to evaluate NOX2 activity. In wt mice platelets, p47phox phosphorylation is enhanced with increasing dose of thrombin, while it is strongly reduced in β3-/- platelets. This is validated by the data showing SFK inhibitor, but not Syk inhibitor was able to prevent p47phox phosphorylation, indicating p47phox was downstream of SFK while not Syk signaling pathway. On the other hand, when CRP was used as agonist, there is only partial reduction in β3-/- platelets, suggesting possible integrin-independent pathway for p47phox. A possible alternate would be ITAM pathway, as both SFK and Syk inhibitor prevented p47phox phosphorylation when the platelets was stimulated by GPVI agonist, CRP (Fig. 23). In addition, PKC as a key kinase downstream of ITAM pathway, was able to completely block p47phox phosphorylation. It was wildly reported in leukocytes, different isoforms of PKC are responsible for p47phox activation by phosphorylate multiple serine sites within AIR domain (Bey et al. 2004, Fontayne et al. 2002). In this study, we show that PKC is important for CRP-induced but not thrombin-

induced p47phox phosphorylation on Ser304. In contrary, PI3K/Akt inhibitors prevents p47phox phosphorylation despite of the involving pathways. Although we could not exclude the possibility PKC isoforms (especially calcium-independent ones) phosphorylate p47phox at other serine sites, lack of phos-Ser304 was a good indicator for loss of p47phox activity, as alanine double mutant of Ser303/304 almost completely abolished NOX activity (Inanami et al. 1998, Hoyal et al. 2003). From these data, we conclude that integrin could regulate p47phox activity by targeting the its phosphorylation site, through PI3K/Akt signaling pathway, thus promoting NOX2 activity and ROS production in platelets. Other pathways, such as GPVI/ITAM pathway could also participate in stimulating NOX2 activity. Since we have demonstrated in the previous paper that NOX-dependent ROS generation modifies platelet activation signals by stimulating Syk/PLCγ2 pathway, it therefore forms a positive feedback that could be important to facilitate platelet activation(Delaney et al. 2016). PKC, as well as PI3K/Akt, are suggested to both playing roles activating p47phox in this pathway, although direct evidences such as PKC or Akt interaction with p47phox, and assessment of kinase inhibition on NOX-dependent ROS production should be provided to further substantiate this conclusion.

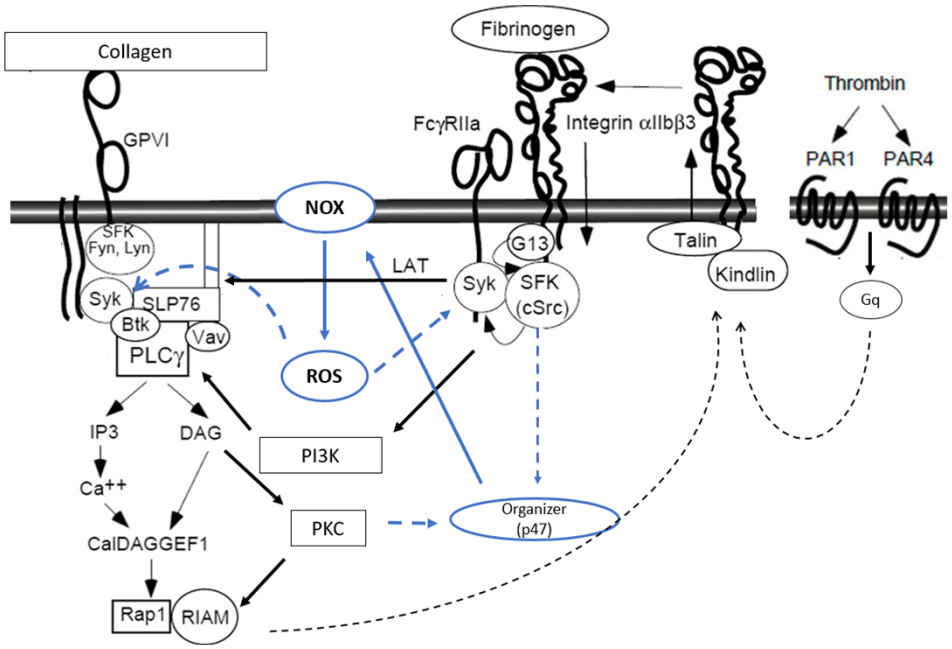


Figure 23. **Diagram of signaling pathways involving ROS production and its effect on platelet activation pathways.** (Adapted from Li *et al*, 2010 with permission.)

Moreover, integrin outside-in signaling is shown to be important for ROS production and NOX2 activation. Data here demonstrated P47phox phosphorylation and ROS production could be induced by integrin-ligation without need for other stimuli, and it is augmented by outside-in signaling activator, MnCl2, while disrupted by selective outside-in inhibitor, mP6. And the absence of β3 is shown to almost completely abolished p47phox activation, even in CRP-stimulated platelets. Importantly, regulation by integrin on NOX2 activity is through activating p47 subunits, which fits the criteria for selective NOX2 inhibitor just mentioned before. It should be noticed that NOXs play important roles in some essential processes such as postural balance, learning and memory, vasodilation, endothelial cell survival, as well as immune defense (Banfi et al. 2004, Paravicini et al. 2004, Paravicini et al. 2006, Kishida et al. 2006, Pao

et al. 2004). One should thus keep precautious when developing therapeutic strategy to avoid disruption of normal NOX-dependent processes. Regulation by integrin is a quite unique features in platelets, and has not yet been identified in cell types where NOX play important functional roles. This should provide another level of selectivity by limiting the inhibitory effect on desired process, by targeting at integrin outside-in signal, thus a compelling strategy for drug intervention.

Lastly, we have demonstrated that NOX1 and NOX2 knockout platelets was defect in stable adhesion, which is not further inhibited by mp6. This could possibly suggest ROS reversely inhibits integrin outside-in signaling. Previous data also have shown that NOX1/2 knockout mainly affected platelet granule secretion, and second wave of platelet aggregation, which requires integrin outside-in signaling (Delaney et al. 2016). However, we here found that platelet spreading is not affected by NOX knockouts. This is conceivable result considering ROS mainly exerting its effect by modulating Syk/PLCγ2 activity, which is not a predominate regulator for platelet spreading.

V. CONCLUSIONS

Excessive ROS is associated with all known cardiovascular risk factors and is believed to play an important role for the onset of cardiovascular diseases. Contrary to this well-known understanding, anti-oxidants have failed to produce efficacy towards preventing cardiovascular events in multiple clinical trials. This could be attributed to poor reactivity of conventional antioxidant with many endogenous ROS, low bioavailability at the site of disease, and many other limitations. Therefore, more effective strategy to eliminate ROS would be targeting the resources where ROS are being generated than scavenging once they are formed, in order to provide cardiovascular benefits.

NADPH oxidase are the key generators of ROS in the blood vessel wall and other tissues during cardiovascular disease progression. Studies from both human and animal platelet has suggested the role of NADPH-dependent ROS in platelet activation and thrombosis.

This study focuses on illustrating the mechanism of how does integrin αIIbβ3 regulate ROS production and NOX activity in platelets. Integrin outside-in signaling is shown to be important for ROS production and NOX2 activation. Data here demonstrated P47phox phosphorylation and ROS production could be induced by integrin-ligation without need for other stimuli, and it is augmented by outside-in signaling activator, $MnCl_2$, while disrupted by selective outside-in inhibitor, mP6. And the absence of β3 is shown to almost completely abolished p47phox activation, even in CRP-stimulated platelets.

In addition, it has demonstrated that NOX1 and NOX2 knockout platelets was defect in stable adhesion, which is not further inhibited by mp6. This could possibly suggest ROS reversely inhibits integrin outside-in signaling. However, platelet spreading is not affected by

NOX knockout. Along with previous data showing that NOX1/2 knockout mainly affected platelet granule secretion, and second wave of platelet aggregation, which requires integrin outside-in signaling (Delaney et al. 2016), we concluded NOX-dependent ROS is important for integrin outside-in signaling and its functional consequences.

Altogether, this study, provided insight as how ROS production in activated platelets is regulated, along with our previous data about ROS effect on platelet activation, forming a theoretical foundation for new antithrombotic strategy basing on intervening integrin outside-in pathway-regulated NOX activity.

CITED LITERATURES

Abtahian, F., N. Bezman, R. Clemens, E. Sebzda, L. Cheng, S. J. Shattil, M. L. Kahn, and G. A. Koretzky. 2006. "Evidence for the requirement of ITAM domains but not SLP-76/Gads interaction for integrin signaling in hematopoietic cells." *Mol Cell Biol* 26 (18):6936-49. doi: 10.1128/mcb.01040-06.

Adachi, T., R. M. Weisbrod, D. R. Pimentel, J. Ying, V. S. Sharov, C. Schoneich, and R. A. Cohen. 2004. "S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide." *Nat Med* 10 (11):1200-7. doi: 10.1038/nm1119.

Ago, T., T. Kitazono, J. Kuroda, Y. Kumai, M. Kamouchi, H. Ooboshi, M. Wakisaka, T. Kawahara, K. Rokutan, S. Ibayashi, and M. Iida. 2005. "NAD(P)H oxidases in rat basilar arterial endothelial cells." *Stroke* 36 (5):1040-6. doi: 10.1161/01.STR.0000163111.05825.0b.

Alexander, B., and G. Landwehr. 1949. "Thrombasthenia and thrombocytopenic purpura; report of case demonstrating qualitative and quantitative inadequacy of platelets." *N Engl J Med* 241 (24):965-8. doi: 10.1056/nejm194912152412403.

Ambasta, R. K., J. G. Schreiber, M. Janiszewski, R. Busse, and R. P. Brandes. 2006. "Noxa1 is a central component of the smooth muscle NADPH oxidase in mice." *Free Radic Biol Med* 41 (2):193-201. doi: 10.1016/j.freeradbiomed.2005.12.035.

Andrews, R. K., and J. E. Fox. 1992. "Identification of a region in the cytoplasmic domain of the platelet membrane glycoprotein Ib-IX complex that binds to purified actin-binding protein." *J Biol Chem* 267 (26):18605-11.

Andrews, R. K., S. J. Harris, T. McNally, and M. C. Berndt. 1998. "Binding of purified 14-3-3 zeta signaling protein to discrete amino acid sequences within the cytoplasmic domain of the platelet membrane glycoprotein Ib-IX-V complex." *Biochemistry* 37 (2):638-47. doi: 10.1021/bi970893g.

Andrews, R. K., J. A. Lopez, and M. C. Berndt. 1997. "Molecular mechanisms of platelet adhesion and activation." *Int J Biochem Cell Biol* 29 (1):91-105.

Andrews, R. K., A. D. Munday, C. A. Mitchell, and M. C. Berndt. 2001. "Interaction of calmodulin with the cytoplasmic domain of the platelet membrane glycoprotein Ib-IX-V complex." *Blood* 98 (3):681-7.

Arias-Salgado, E. G., F. Haj, C. Dubois, B. Moran, A. Kasirer-Friede, B. C. Furie, B. Furie, B. G. Neel, and S. J. Shattil. 2005. "PTP-1B is an essential positive regulator of platelet integrin signaling." *J Cell Biol* 170 (5):837-45. doi: 10.1083/jcb.200503125.

Arias-Salgado, E. G., S. Lizano, S. Sarkar, J. S. Brugge, M. H. Ginsberg, and S. J. Shattil. 2003. "Src kinase activation by direct interaction with the integrin beta cytoplasmic domain." *Proc Natl Acad Sci U S A* 100 (23):13298-302. doi: 10.1073/pnas.2336149100.

Arthur, J. F., J. Qiao, Y. Shen, A. K. Davis, E. Dunne, M. C. Berndt, E. E. Gardiner, and R. K. Andrews. 2012. "ITAM receptor-mediated generation of reactive oxygen species in human platelets occurs via Syk-dependent and Syk-independent pathways." *J Thromb Haemost* 10 (6):1133-41.

Arthur, W. T., and K. Burridge. 2001. "RhoA inactivation by p190RhoGAP regulates cell spreading and migration by promoting membrane protrusion and polarity." *Mol Biol Cell* 12 (9):2711-20.

Babior, B. M. 1999. "NADPH oxidase: an update." *Blood* 93 (5):1464-76.

Back, L. D., J. R. Radbill, and D. W. Crawford. 1977. "Analysis of pulsatile, viscous blood flow through diseased coronary arteries of man." *J Biomech* 10 (5-6):339-53.

Banfi, B., R. A. Clark, K. Steger, and K. H. Krause. 2003. "Two novel proteins activate superoxide generation by the NADPH oxidase NOX1." *J Biol Chem* 278 (6):3510-3. doi: 10.1074/jbc.C200613200.

Banfi, B., B. Malgrange, J. Knisz, K. Steger, M. Dubois-Dauphin, and K. H. Krause. 2004. "NOX3, a superoxide-generating NADPH oxidase of the inner ear." *J Biol Chem* 279 (44):46065-72. doi: 10.1074/jbc.M403046200.

Banfi, B., A. Maturana, S. Jaconi, S. Arnaudeau, T. Laforge, B. Sinha, E. Ligeti, N. Demaurex, and K. H. Krause. 2000. "A mammalian H+ channel generated through alternative splicing of the NADPH oxidase homolog NOH-1." *Science* 287 (5450):138-42.

Barford, D. 2004. "The role of cysteine residues as redox-sensitive regulatory switches." *Curr Opin Struct Biol* 14 (6):679-86. doi: 10.1016/j.sbi.2004.09.012.

Beckman, K. B., and B. N. Ames. 1998. "The free radical theory of aging matures." *Physiol Rev* 78 (2):547-81.

Bergmeier, W., C. L. Piffath, T. Goerge, S. M. Cifuni, Z. M. Ruggeri, J. Ware, and D. D. Wagner. 2006. "The role of platelet adhesion receptor GPIbalpha far exceeds that of its main ligand, von Willebrand factor, in arterial thrombosis." *Proc Natl Acad Sci U S A* 103 (45):16900-5. doi: 10.1073/pnas.0608207103.

Berndt, M. C., Y. Shen, S. M. Dopheide, E. E. Gardiner, and R. K. Andrews. 2001. "The vascular biology of the glycoprotein Ib-IX-V complex." *Thromb Haemost* 86 (1):178-88.

Berndt, M. C., C. M. Ward, W. J. Booth, P. A. Castaldi, A. V. Mazurov, and R. K. Andrews. 1992. "Identification of aspartic acid 514 through glutamic acid 542 as a glycoprotein Ib-IX

complex receptor recognition sequence in von Willebrand factor. Mechanism of modulation of von Willebrand factor by ristocetin and botrocetin." *Biochemistry* 31 (45):11144-51.

Bey, E. A., B. Xu, A. Bhattacharjee, C. M. Oldfield, X. Zhao, Q. Li, V. Subbulakshmi, G. M. Feldman, F. B. Wientjes, and M. K. Cathcart. 2004. "Protein kinase C delta is required for p47phox phosphorylation and translocation in activated human monocytes." *J Immunol* 173 (9):5730-8.

Bleys, J., E. R. Miller, 3rd, R. Pastor-Barriuso, L. J. Appel, and E. Guallar. 2006. "Vitamin-mineral supplementation and the progression of atherosclerosis: a meta-analysis of randomized controlled trials." *Am J Clin Nutr* 84 (4):880-7; quiz 954-5.

Bodnar, R. J., M. Gu, Z. Li, G. D. Englund, and X. Du. 1999. "The cytoplasmic domain of the platelet glycoprotein Ibalpha is phosphorylated at serine 609." *J Biol Chem* 274 (47):33474-9.

Boylan, B., C. Gao, V. Rathore, J. C. Gill, D. K. Newman, and P. J. Newman. 2008. "Identification of FcgammaRIIa as the ITAM-bearing receptor mediating alphaIIbbeta3 outside-in integrin signaling in human platelets." *Blood* 112 (7):2780-6. doi: 10.1182/blood-2008-02-142125.

Bunting, M., E. S. Harris, T. M. McIntyre, S. M. Prescott, and G. A. Zimmerman. 2002. "Leukocyte adhesion deficiency syndromes: adhesion and tethering defects involving beta 2 integrins and selectin ligands." *Curr Opin Hematol* 9 (1):30-5.

Cadenas, E., and K. J. Davies. 2000. "Mitochondrial free radical generation, oxidative stress, and aging." *Free Radic Biol Med* 29 (3-4):222-30.

Caen, J. P., S. Cronberg, S. Levy-Toledano, P. Kubisz, and J. P. Pinkhas. 1971. "New data on Glanzmann's thrombasthenia." *Proc Soc Exp Biol Med* 136 (4):1082-6.

Calderwood, D. A., R. Zent, R. Grant, D. J. Rees, R. O. Hynes, and M. H. Ginsberg. 1999. "The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation." *J Biol Chem* 274 (40):28071-4.

Canobbio, I., C. Balduini, and M. Torti. 2004. "Signalling through the platelet glycoprotein Ib-V-IX complex." *Cell Signal* 16 (12):1329-44. doi: 10.1016/j.cellsig.2004.05.008.

Cauwenberghs, N., K. Vanhoorelbeke, S. Vauterin, D. F. Westra, G. Romo, E. G. Huizinga, J. A. Lopez, M. C. Berndt, J. Harsfalvi, and H. Deckmyn. 2001. "Epitope mapping of inhibitory antibodies against platelet glycoprotein Ibalpha reveals interaction between the leucine-rich repeat N-terminal and C-terminal flanking domains of glycoprotein Ibalpha." *Blood* 98 (3):652-60.

Chen, J., S. De, D. S. Damron, W. S. Chen, N. Hay, and T. V. Byzova. 2004. "Impaired platelet responses to thrombin and collagen in AKT-1-deficient mice." *Blood* 104 (6):1703-10. doi: 10.1182/blood-2003-10-3428.

Chen, J., T. G. Diacovo, D. G. Grenache, S. A. Santoro, and M. M. Zutter. 2002. "The alpha(2) integrin subunit-deficient mouse: a multifaceted phenotype including defects of branching morphogenesis and hemostasis." *Am J Pathol* 161 (1):337-44.

Cheng, G., B. A. Diebold, Y. Hughes, and J. D. Lambeth. 2006. "Nox1-dependent reactive oxygen generation is regulated by Rac1." *J Biol Chem* 281 (26):17718-26. doi: 10.1074/jbc.M512751200.

Cheng, G., and J. D. Lambeth. 2004. "NOXO1, regulation of lipid binding, localization, and activation of Nox1 by the Phox homology (PX) domain." *J Biol Chem* 279 (6):4737-42. doi: 10.1074/jbc.M305968200.

Cheranov, S. Y., and J. H. Jaggar. 2006. "TNF-alpha dilates cerebral arteries via NAD(P)H oxidase-dependent Ca2+ spark activation." *Am J Physiol Cell Physiol* 290 (4):C964-71. doi: 10.1152/ajpcell.00499.2005.

Chiarugi, P., G. Pani, E. Giannoni, L. Taddei, R. Colavitti, G. Raugei, M. Symons, S. Borrello, T. Galeotti, and G. Ramponi. 2003. "Reactive oxygen species as essential mediators of cell adhesion: the oxidative inhibition of a FAK tyrosine phosphatase is required for cell adhesion." *J Cell Biol* 161 (5):933-44. doi: 10.1083/jcb.200211118.

Choi, W. S., W. J. Rice, D. L. Stokes, and B. S. Coller. 2013. "Three-dimensional reconstruction of intact human integrin alphaIIbbeta3: new implications for activation-dependent ligand binding." *Blood* 122 (26):4165-71. doi: 10.1182/blood-2013-04-499194.

Chrzanowska-Wodnicka, M., S. S. Smyth, S. M. Schoenwaelder, T. H. Fischer, and G. C. White, 2nd. 2005. "Rap1b is required for normal platelet function and hemostasis in mice." *J Clin Invest* 115 (3):680-7. doi: 10.1172/jci22973.

Clemetson, J. M., J. Polgar, E. Magnenat, T. N. Wells, and K. J. Clemetson. 1999. "The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcalphaR and the natural killer receptors." *J Biol Chem* 274 (41):29019-24.

Clemetson, K. J., and J. M. Clemetson. 2001. "Platelet Collagen Receptors." *Thrombosis and Haemostasis* 86 (7):189-97.

Coughlin, S. R. 2005. "Protease-activated receptors in hemostasis, thrombosis and vascular biology." *J Thromb Haemost* 3 (8):1800-14. doi: 10.1111/j.1538-7836.2005.01377.x.

Covic, L., A. L. Gresser, and A. Kuliopulos. 2000. "Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets." *Biochemistry* 39 (18):5458-67.

Crofford, L. J. 1997. "COX-1 and COX-2 tissue expression: implications and predictions." *J Rheumatol Suppl* 49:15-9.

Dai, K., R. Bodnar, M. C. Berndt, and X. Du. 2005. "A critical role for 14-3-3zeta protein in regulating the VWF binding function of platelet glycoprotein Ib-IX and its therapeutic implications." *Blood* 106 (6):1975-81. doi: 10.1182/blood-2005-01-0440.

Daimon, T., and Y. Gotoh. 1982. "Cytochemical evidence of the origin of the dense tubular system in the mouse platelet." *Histochemistry* 76 (2):189-96.

Daniel, J. L., C. Dangelmaier, J. Jin, B. Ashby, J. B. Smith, and S. P. Kunapuli. 1998. "Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets." *J Biol Chem* 273 (4):2024-9.

De Candia, E., S. W. Hall, S. Rutella, R. Landolfi, R. K. Andrews, and R. De Cristofaro. 2001. "Binding of thrombin to glycoprotein Ib accelerates the hydrolysis of Par-1 on intact platelets." *J Biol Chem* 276 (7):4692-8. doi: 10.1074/jbc.M008160200.

De Cristofaro, R., E. De Candia, S. Rutella, and J. I. Weitz. 2000. "The Asp(272)-Glu(282) region of platelet glycoprotein Ibalpha interacts with the heparin-binding site of alpha-thrombin and protects the enzyme from the heparin-catalyzed inhibition by antithrombin III." *J Biol Chem* 275 (6):3887-95.

de Larouziere, V., J. P. Brouland, F. Souni, L. Drouet, and E. Cramer. 1998. "Inverse immunostaining pattern for synthesized versus endocytosed alpha-granule proteins in human bone marrow megakaryocytes." *Br J Haematol* 101 (4):618-25.

De Marco, L., M. Mazzucato, A. Masotti, J. W. Fenton, 2nd, and Z. M. Ruggeri. 1991. "Function of glycoprotein Ib alpha in platelet activation induced by alpha-thrombin." *J Biol Chem* 266 (35):23776-83.

de Virgilio, M., W. B. Kiosses, and S. J. Shattil. 2004. "Proximal, selective, and dynamic interactions between integrin alphaIIbbeta3 and protein tyrosine kinases in living cells." *J Cell Biol* 165 (3):305-11. doi: 10.1083/jcb.200402064.

Delaney, M. K., K. Kim, B. Estevez, Z. Xu, A. Stojanovic-Terpo, B. Shen, M. Ushio-Fukai, J. Cho, and X. Du. 2016. "Differential Roles of the NADPH-Oxidase 1 and 2 in Platelet Activation and Thrombosis." *Arterioscler Thromb Vasc Biol* 36 (5):846-54. doi: 10.1161/atvbaha.116.307308.

Didichenko, S. A., B. Tilton, B. A. Hemmings, K. Ballmer-Hofer, and M. Thelen. 1996. "Constitutive activation of protein kinase B and phosphorylation of p47phox by a membrane-targeted phosphoinositide 3-kinase." *Curr Biol* 6 (10):1271-8.

Dinauer, M. C., E. A. Pierce, G. A. Bruns, J. T. Curnutte, and S. H. Orkin. 1990. "Human neutrophil cytochrome b light chain (p22-phox). Gene structure, chromosomal location, and

mutations in cytochrome-negative autosomal recessive chronic granulomatous disease." *J Clin Invest* 86 (5):1729-37. doi: 10.1172/jci114898.

Ding, J., C. J. Vlahos, R. Liu, R. F. Brown, and J. A. Badwey. 1995. "Antagonists of phosphatidylinositol 3-kinase block activation of several novel protein kinases in neutrophils." *J Biol Chem* 270 (19):11684-91.

Djellas, Y., J. M. Manganello, K. Antonakis, and G. C. Le Breton. 1999. "Identification of Galpha13 as one of the G-proteins that couple to human platelet thromboxane A2 receptors." *J Biol Chem* 274 (20):14325-30.

Djordjevic, T., A. Pogrebniak, R. S. BelAiba, S. Bonello, C. Wotzlaw, H. Acker, J. Hess, and A. Gorlach. 2005. "The expression of the NADPH oxidase subunit p22phox is regulated by a redox-sensitive pathway in endothelial cells." *Free Radic Biol Med* 38 (5):616-30. doi: 10.1016/j.freeradbiomed.2004.09.036.

Dong, J. F., C. Q. Li, and J. A. Lopez. 1994. "Tyrosine sulfation of the glycoprotein Ib-IX complex: identification of sulfated residues and effect on ligand binding." *Biochemistry* 33 (46):13946-53.

Dong, J. F., G. Sae-Tung, and J. A. Lopez. 1997. "Role of glycoprotein V in the formation of the platelet high-affinity thrombin-binding site." *Blood* 89 (12):4355-63.

Dorn, G. W., 2nd. 1989. "Distinct platelet thromboxane A2/prostaglandin H2 receptor subtypes. A radioligand binding study of human platelets." *J Clin Invest* 84 (6):1883-91. doi: 10.1172/jci114375.

Dorsam, R. T., and S. P. Kunapuli. 2004. "Central role of the P2Y12 receptor in platelet activation." *J Clin Invest* 113 (3):340-5. doi: 10.1172/jci20986.

Drummond, G. R., S. Selemidis, K. K. Griendling, and C. G. Sobey. 2011. "Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets." *Nat Rev Drug Discov* 10 (6):453-71. doi: 10.1038/nrd3403.

Du, X. 2007. "Signaling and regulation of the platelet glycoprotein Ib-IX-V complex." *Curr Opin Hematol* 14 (3):262-9. doi: 10.1097/MOH.0b013e3280dce51a.

Du, X., J. E. Fox, and S. Pei. 1996. "Identification of a binding sequence for the 14-3-3 protein within the cytoplasmic domain of the adhesion receptor, platelet glycoprotein Ib alpha." *J Biol Chem* 271 (13):7362-7.

Du, X., T. C. Saido, S. Tsubuki, F. E. Indig, M. J. Williams, and M. H. Ginsberg. 1995. "Calpain cleavage of the cytoplasmic domain of the integrin beta 3 subunit." *J Biol Chem* 270 (44):26146-51.

Dubois, R. N., S. B. Abramson, L. Crofford, R. A. Gupta, L. S. Simon, L. B. Van De Putte, and P. E. Lipsky. 1998. "Cyclooxygenase in biology and disease." *Faseb j* 12 (12):1063-73.

Ebbe, Sherley, Frederick Stohlman, Janet Donovan, and Donald Howard. 1965. "Megakaryocytopoiesis in the Rat." *Blood* 26 (1):20-35.

El Benna, J., R. P. Faust, J. L. Johnson, and B. M. Babior. 1996. "Phosphorylation of the respiratory burst oxidase subunit p47phox as determined by two-dimensional phosphopeptide mapping. Phosphorylation by protein kinase C, protein kinase A, and a mitogen-activated protein kinase." *J Biol Chem* 271 (11):6374-8.

Escolar, G., and J. G. White. 1991. "The platelet open canalicular system: a final common pathway." *Blood Cells* 17 (3):467-85; discussion 486-95.

Estevez, B., K. Kim, M. K. Delaney, A. Stojanovic-Terpo, B. Shen, C. Ruan, J. Cho, Z. M. Ruggeri, and X. Du. 2015. "Signaling-mediated cooperativity between glycoprotein Ib-IX and protease-activated receptors in thrombin-induced platelet activation." *Blood*. doi: 10.1182/blood-2015-04-638387.

Ezumi, Y., K. Shindoh, M. Tsuji, and H. Takayama. 1998. "Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor glycoprotein VI-Fc receptor gamma chain complex on human platelets." *J Exp Med* 188 (2):267-76.

Fabre, J. E., M. Nguyen, A. Latour, J. A. Keifer, L. P. Audoly, T. M. Coffman, and B. H. Koller. 1999. "Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice." *Nat Med* 5 (10):1199-202. doi: 10.1038/13522.

Faust, L. R., J. el Benna, B. M. Babior, and S. J. Chanock. 1995. "The phosphorylation targets of p47phox, a subunit of the respiratory burst oxidase. Functions of the individual target serines as evaluated by site-directed mutagenesis." *Journal of Clinical Investigation* 96 (3):1499-1505.

Feng, Y., and C. A. Walsh. 2004. "The many faces of filamin: a versatile molecular scaffold for cell motility and signalling." *Nat Cell Biol* 6 (11):1034-8. doi: 10.1038/ncb1104-1034.

Flevaris, P., A. Stojanovic, H. Gong, A. Chishti, E. Welch, and X. Du. 2007. "A molecular switch that controls cell spreading and retraction." *J Cell Biol* 179 (3):553-65. doi: 10.1083/jcb.200703185.

Fontayne, Alexandre, Pham My-Chan Dang, Marie-Anne Gougerot-Pocidalo, and Jamel El Benna. 2002. "Phosphorylation of p47phox Sites by PKC α, βII, δ, and ζ: Effect on Binding to p22phox and on NADPH Oxidase Activation." *Biochemistry* 41 (24):7743-7750. doi: 10.1021/bi011953s.

Foster, C. J., D. M. Prosser, J. M. Agans, Y. Zhai, M. D. Smith, J. E. Lachowicz, F. L. Zhang, E. Gustafson, F. J. Monsma, Jr., M. T. Wiekowski, S. J. Abbondanzo, D. N. Cook, M. L. Bayne, S. A. Lira, and M. S. Chintala. 2001. "Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs." *J Clin Invest* 107 (12):1591-8. doi: 10.1172/jci12242.

Fox, J. E. 2001. "Cytoskeletal proteins and platelet signaling." *Thromb Haemost* 86 (1):198-213.

Fox, J. E., R. G. Taylor, M. Taffarel, J. K. Boyles, and D. E. Goll. 1993. "Evidence that activation of platelet calpain is induced as a consequence of binding of adhesive ligand to the integrin, glycoprotein IIb-IIIa." *J Cell Biol* 120 (6):1501-7.

Fox, James G. 2007. *The mouse in biomedical research, 2nd ed*. Amsterdam, Boston: Elsevier.

Fredrickson, B. J., J. F. Dong, L. V. McIntire, and J. A. Lopez. 1998. "Shear-dependent rolling on von Willebrand factor of mammalian cells expressing the platelet glycoprotein Ib-IX-V complex." *Blood* 92 (10):3684-93.

Frungieri, M. B., S. Weidinger, V. Meineke, F. M. Kohn, and A. Mayerhofer. 2002. "Proliferative action of mast-cell tryptase is mediated by PAR2, COX2, prostaglandins, and PPARgamma : Possible relevance to human fibrotic disorders." *Proc Natl Acad Sci U S A* 99 (23):15072-7. doi: 10.1073/pnas.232422999.

Furst, R., C. Brueckl, W. M. Kuebler, S. Zahler, F. Krotz, A. Gorlach, A. M. Vollmar, and A. K. Kiemer. 2005. "Atrial natriuretic peptide induces mitogen-activated protein kinase phosphatase-1 in human endothelial cells via Rac1 and NAD(P)H oxidase/Nox2-activation." *Circ Res* 96 (1):43-53. doi: 10.1161/01.res.0000151983.01148.06.

Gao, J., K. E. Zoller, M. H. Ginsberg, J. S. Brugge, and S. J. Shattil. 1997. "Regulation of the pp72syk protein tyrosine kinase by platelet integrin alpha IIb beta 3." *Embo j* 16 (21):6414-25. doi: 10.1093/emboj/16.21.6414.

Gautier, P., and J. Guinand-Doniol. 1952. "[A new syndrome; Lobstein's disease associated with Glanzmann's familial and hereditary thrombasthenia]." *Schweiz Med Wochenschr* 82 (15):407-9.

Gavazzi, G., B. Banfi, C. Deffert, L. Fiette, M. Schappi, F. Herrmann, and K. H. Krause. 2006. "Decreased blood pressure in NOX1-deficient mice." *FEBS Lett* 580 (2):497-504. doi: 10.1016/j.febslet.2005.12.049.

Geiszt, M., J. B. Kopp, P. Varnai, and T. L. Leto. 2000. "Identification of renox, an NAD(P)H oxidase in kidney." *Proc Natl Acad Sci U S A* 97 (14):8010-4. doi: 10.1073/pnas.130135897.

Geiszt, M., K. Lekstrom, J. Witta, and T. L. Leto. 2003. "Proteins homologous to p47phox and p67phox support superoxide production by NAD(P)H oxidase 1 in colon epithelial cells." *J Biol Chem* 278 (22):20006-12. doi: 10.1074/jbc.M301289200.

Gibbins, J. M., M. Okuma, R. Farndale, M. Barnes, and S. P. Watson. 1997. "Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor gamma-chain." *FEBS Lett* 413 (2):255-9.

Ginsberg, M. H., J. Forsyth, A. Lightsey, J. Chediak, and E. F. Plow. 1983. "Reduced surface expression and binding of fibronectin by thrombin-stimulated thrombasthenic platelets." *J Clin Invest* 71 (3):619-24.

Gong, H., B. Shen, P. Flevaris, C. Chow, S. C. Lam, T. A. Voyno-Yasenetskaya, T. Kozasa, and X. Du. 2010. "G protein subunit Galpha13 binds to integrin alphaIIbbeta3 and mediates integrin "outside-in" signaling." *Science* 327 (5963):340-3. doi: 10.1126/science.1174779.

Granados, M. P., G. M. Salido, A. Gonzalez, and J. A. Pariente. 2006. "Dose-dependent effect of hydrogen peroxide on calcium mobilization in mouse pancreatic acinar cells." *Biochem Cell Biol* 84 (1):39-48. doi: 10.1139/o05-150.

Griendling, K. K., D. Sorescu, B. Lassegue, and M. Ushio-Fukai. 2000. "Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology." *Arterioscler Thromb Vasc Biol* 20 (10):2175-83.

Haimovich, B., L. Lipfert, J. S. Brugge, and S. J. Shattil. 1993. "Tyrosine phosphorylation and cytoskeletal reorganization in platelets are triggered by interaction of integrin receptors with their immobilized ligands." *J Biol Chem* 268 (21):15868-77.

Haling, J. R., S. J. Monkley, D. R. Critchley, and B. G. Petrich. 2011. "Talin-dependent integrin activation is required for fibrin clot retraction by platelets." *Blood* 117 (5):1719-22. doi: 10.1182/blood-2010-09-305433.

Hall, A. 2005. "Rho GTPases and the control of cell behaviour." *Biochem Soc Trans* 33 (Pt 5):891-5. doi: 10.1042/bst20050891.

Harburger, D. S., M. Bouaouina, and D. A. Calderwood. 2009. "Kindlin-1 and -2 directly bind the C-terminal region of beta integrin cytoplasmic tails and exert integrin-specific activation effects." *J Biol Chem* 284 (17):11485-97. doi: 10.1074/jbc.M809233200.

Hartwig, J. H. 1992. "Mechanisms of actin rearrangements mediating platelet activation." *J Cell Biol* 118 (6):1421-42.

Hechler, B., N. Lenain, P. Marchese, C. Vial, V. Heim, M. Freund, J. P. Cazenave, M. Cattaneo, Z. M. Ruggeri, R. Evans, and C. Gachet. 2003. "A role of the fast ATP-gated P2X1 cation

channel in thrombosis of small arteries in vivo." *J Exp Med* 198 (4):661-7. doi: 10.1084/jem.20030144.

Hers, I., J. Donath, G. van Willigen, and J. W. Akkerman. 1998. "Differential involvement of tyrosine and serine/threonine kinases in platelet integrin alphaIIbbeta3 exposure." *Arterioscler Thromb Vasc Biol* 18 (3):404-14.

Hickey, M. J., F. S. Hagen, M. Yagi, and G. J. Roth. 1993. "Human platelet glycoprotein V: characterization of the polypeptide and the related Ib-V-IX receptor system of adhesive, leucine-rich glycoproteins." *Proc Natl Acad Sci U S A* 90 (18):8327-31.

Hickey, M. J., S. A. Williams, and G. J. Roth. 1989. "Human platelet glycoprotein IX: an adhesive prototype of leucine-rich glycoproteins with flank-center-flank structures." *Proc Natl Acad Sci U S A* 86 (17):6773-7.

Hollopeter, G., H. M. Jantzen, D. Vincent, G. Li, L. England, V. Ramakrishnan, R. B. Yang, P. Nurden, A. Nurden, D. Julius, and P. B. Conley. 2001. "Identification of the platelet ADP receptor targeted by antithrombotic drugs." *Nature* 409 (6817):202-7. doi: 10.1038/35051599.

Honore, S., H. Kovacic, V. Pichard, C. Briand, and J. B. Rognoni. 2003. "Alpha2beta1-integrin signaling by itself controls G1/S transition in a human adenocarcinoma cell line (Caco-2): implication of NADPH oxidase-dependent production of ROS." *Exp Cell Res* 285 (1):59-71.

Hotchkiss, R. S., and I. E. Karl. 2003. "The pathophysiology and treatment of sepsis." *N Engl J Med* 348 (2):138-50. doi: 10.1056/NEJMra021333.

Hoyal, C. R., A. Gutierrez, B. M. Young, S. D. Catz, J. H. Lin, P. N. Tsichlis, and B. M. Babior. 2003. "Modulation of p47PHOX activity by site-specific phosphorylation: Akt-dependent activation of the NADPH oxidase." *Proc Natl Acad Sci U S A* 100 (9):5130-5. doi: 10.1073/pnas.1031526100.

Huttenlocher, A., and A. R. Horwitz. 2011. "Integrins in cell migration." *Cold Spring Harb Perspect Biol* 3 (9):a005074. doi: 10.1101/cshperspect.a005074.

Hynes, R. O. 2002. "Integrins: bidirectional, allosteric signaling machines." *Cell* 110 (6):673-87.

Inanami, O., J. L. Johnson, J. K. McAdara, J. E. Benna, L. R. Faust, P. E. Newburger, and B. M. Babior. 1998. "Activation of the leukocyte NADPH oxidase by phorbol ester requires the phosphorylation of p47PHOX on serine 303 or 304." *J Biol Chem* 273 (16):9539-43.

Ishii, K., R. Gerszten, Y. W. Zheng, J. B. Welsh, C. W. Turck, and S. R. Coughlin. 1995. "Determinants of thrombin receptor cleavage. Receptor domains involved, specificity, and role of the P3 aspartate." *J Biol Chem* 270 (27):16435-40.

Italiano, J. E., Jr., P. Lecine, R. A. Shivdasani, and J. H. Hartwig. 1999. "Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes." *J Cell Biol* 147 (6):1299-312.

Jantzen, H. M., D. S. Milstone, L. Gousset, P. B. Conley, and R. M. Mortensen. 2001. "Impaired activation of murine platelets lacking G alpha(i2)." *J Clin Invest* 108 (3):477-83. doi: 10.1172/jci12818.

Jenkins, A. L., L. Nannizzi-Alaimo, D. Silver, J. R. Sellers, M. H. Ginsberg, D. A. Law, and D. R. Phillips. 1998. "Tyrosine phosphorylation of the beta3 cytoplasmic domain mediates integrin-cytoskeletal interactions." *J Biol Chem* 273 (22):13878-85.

Jin, J., and S. P. Kunapuli. 1998. "Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation." *Proc Natl Acad Sci U S A* 95 (14):8070-4.

Jirouskova, M., J. K. Jaiswal, and B. S. Coller. 2007. "Ligand density dramatically affects integrin alpha IIb beta 3-mediated platelet signaling and spreading." *Blood* 109 (12):5260-9. doi: 10.1182/blood-2006-10-054015.

Kahn, M. L., M. Nakanishi-Matsui, M. J. Shapiro, H. Ishihara, and S. R. Coughlin. 1999. "Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin." *J Clin Invest* 103 (6):879-87. doi: 10.1172/jci6042.

Kahn, M. L., Y. W. Zheng, W. Huang, V. Bigornia, D. Zeng, S. Moff, R. V. Farese, Jr., C. Tam, and S. R. Coughlin. 1998. "A dual thrombin receptor system for platelet activation." *Nature* 394 (6694):690-4. doi: 10.1038/29325.

Kandel, E. S., and N. Hay. 1999. "The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB." *Exp Cell Res* 253 (1):210-29. doi: 10.1006/excr.1999.4690.

Kanofsky, J. R. 1989. "Singlet oxygen production by biological systems." *Chem Biol Interact* 70 (1-2):1-28.

Kasirer-Friede, A., M. R. Cozzi, M. Mazzucato, L. De Marco, Z. M. Ruggeri, and S. J. Shattil. 2004. "Signaling through GP Ib-IX-V activates alpha IIb beta 3 independently of other receptors." *Blood* 103 (9):3403-11. doi: 10.1182/blood-2003-10-3664.

Katayama, T., Y. Ikeda, M. Handa, T. Tamatani, S. Sakamoto, M. Ito, Y. Ishimura, and M. Suematsu. 2000. "Immunoneutralization of glycoprotein Ibalpha attenuates endotoxin-induced interactions of platelets and leukocytes with rat venular endothelium in vivo." *Circ Res* 86 (10):1031-7.

Kenny, D., P. A. Morateck, and R. R. Montgomery. 2002. "The cysteine knot of platelet glycoprotein Ib beta (GPIb beta) is critical for the interaction of GPIb beta with GPIX." *Blood* 99 (12):4428-33.

Kim, S., C. Foster, A. Lecchi, T. M. Quinton, D. M. Prosser, J. Jin, M. Cattaneo, and S. P. Kunapuli. 2002. "Protease-activated receptors 1 and 4 do not stimulate G(i) signaling pathways in the absence of secreted ADP and cause human platelet aggregation independently of G(i) signaling." *Blood* 99 (10):3629-36.

Kimura, K., M. Ito, M. Amano, K. Chihara, Y. Fukata, M. Nakafuku, B. Yamamori, J. Feng, T. Nakano, K. Okawa, A. Iwamatsu, and K. Kaibuchi. 1996. "Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase)." *Science* 273 (5272):245-8.

Kishida, K. T., C. A. Hoeffer, D. Hu, M. Pao, S. M. Holland, and E. Klann. 2006. "Synaptic plasticity deficits and mild memory impairments in mouse models of chronic granulomatous disease." *Mol Cell Biol* 26 (15):5908-20. doi: 10.1128/mcb.00269-06.

Kishimoto, T. K., K. O'Connor, A. Lee, T. M. Roberts, and T. A. Springer. 1987. "Cloning of the beta subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family." *Cell* 48 (4):681-90.

Klages, B., U. Brandt, M. I. Simon, G. Schultz, and S. Offermanns. 1999. "Activation of G12/G13 results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets." *J Cell Biol* 144 (4):745-54.

Knezevic, I., C. Borg, and G. C. Le Breton. 1993. "Identification of Gq as one of the G-proteins which copurify with human platelet thromboxane A2/prostaglandin H2 receptors." *J Biol Chem* 268 (34):26011-7.

Kroll, M. H., T. S. Harris, J. L. Moake, R. I. Handin, and A. I. Schafer. 1991. "von Willebrand factor binding to platelet GpIb initiates signals for platelet activation." *J Clin Invest* 88 (5):1568-73. doi: 10.1172/jci115468.

Kroll, M. H., J. D. Hellums, L. V. McIntire, A. I. Schafer, and J. L. Moake. 1996. "Platelets and shear stress." *Blood* 88 (5):1525-41.

Kroner, C., K. Eybrechts, and J. W. Akkerman. 2000. "Dual regulation of platelet protein kinase B." *J Biol Chem* 275 (36):27790-8. doi: 10.1074/jbc.M000540200.

Krötz, Florian, Hae Young Sohn, Torsten Gloe, Stefan Zahler, Tobias Riexinger, Thomas M. Schiele, Bernhard F. Becker, Karl Theisen, Volker Klauss, and Ulrich Pohl. 2002. "NAD(P)H oxidase–dependent platelet superoxide anion release increases platelet recruitment." *Blood* 100 (3):917-924. doi: 10.1182/blood.V100.3.917.

Kuijpers, M. J., V. Schulte, W. Bergmeier, T. Lindhout, C. Brakebusch, S. Offermanns, R. Fassler, J. W. Heemskerk, and B. Nieswandt. 2003. "Complementary roles of glycoprotein VI and alpha2beta1 integrin in collagen-induced thrombus formation in flowing whole blood ex vivo." *Faseb j* 17 (6):685-7. doi: 10.1096/fj.02-0381fje.

Kumar, Parveen J, and Michael L. Clark. 2012. *Kumar & Clark clinical medicine, 8th ed*. Edinburgh, New York Elsevier Saunders.

Lagarde, M. 1988. "Metabolism of fatty acids by platelets and the functions of various metabolites in mediating platelet function." *Prog Lipid Res* 27 (2):135-52.

Lambeth, J. D., G. Cheng, R. S. Arnold, and W. A. Edens. 2000. "Novel homologs of gp91phox." *Trends Biochem Sci* 25 (10):459-61.

Lammle, B., J. A. Kremer Hovinga, and L. Alberio. 2005. "Thrombotic thrombocytopenic purpura." *J Thromb Haemost* 3 (8):1663-75. doi: 10.1111/j.1538-7836.2005.01425.x.

Lapouge, K., S. J. Smith, P. A. Walker, S. J. Gamblin, S. J. Smerdon, and K. Rittinger. 2000. "Structure of the TPR domain of p67phox in complex with Rac.GTP." *Mol Cell* 6 (4):899-907.

Lassegue, B., D. Sorescu, K. Szocs, Q. Yin, M. Akers, Y. Zhang, S. L. Grant, J. D. Lambeth, and K. K. Griendling. 2001. "Novel gp91(phox) homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways." *Circ Res* 88 (9):888-94.

Lawlor, M. A., and D. R. Alessi. 2001. "PKB/Akt: a key mediator of cell proliferation, survival and insulin responses?" *J Cell Sci* 114 (Pt 16):2903-10.

Lee, H. S., C. J. Lim, W. Puzon-McLaughlin, S. J. Shattil, and M. H. Ginsberg. 2009. "RIAM activates integrins by linking talin to ras GTPase membrane-targeting sequences." *J Biol Chem* 284 (8):5119-27. doi: 10.1074/jbc.M807117200.

Lee, S. R., K. S. Kwon, S. R. Kim, and S. G. Rhee. 1998. "Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor." *J Biol Chem* 273 (25):15366-72.

Leon, C., B. Hechler, M. Freund, A. Eckly, C. Vial, P. Ohlmann, A. Dierich, M. LeMeur, J. P. Cazenave, and C. Gachet. 1999. "Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice." *J Clin Invest* 104 (12):1731-7. doi: 10.1172/jci8399.

Leon, C., C. Vial, C. Gachet, P. Ohlmann, B. Hechler, J. P. Cazenave, A. Lecchi, and M. Cattaneo. 1999. "The P2Y1 receptor is normal in a patient presenting a severe deficiency of ADP-induced platelet aggregation." *Thromb Haemost* 81 (5):775-81.

Li, A., J. Segui, S. H. Heinemann, and T. Hoshi. 1998. "Oxidation regulates cloned neuronal voltage-dependent Ca2+ channels expressed in Xenopus oocytes." *J Neurosci* 18 (17):6740-7.

Li, C. Q., J. F. Dong, F. Lanza, D. A. Sanan, G. Sae-Tung, and J. A. Lopez. 1995. "Expression of platelet glycoprotein (GP) V in heterologous cells and evidence for its association with GP Ib alpha in forming a GP Ib-IX-V complex on the cell surface." *J Biol Chem* 270 (27):16302-7.

Li, Z., M. K. Delaney, K. A. O'Brien, and X. Du. 2010. "Signaling during platelet adhesion and activation." *Arterioscler Thromb Vasc Biol* 30 (12):2341-9. doi: 10.1161/ATVBAHA.110.207522.

Li, Z., X. Xi, and X. Du. 2001. "A mitogen-activated protein kinase-dependent signaling pathway in the activation of platelet integrin alpha IIbbeta3." *J Biol Chem* 276 (45):42226-32. doi: 10.1074/jbc.M106129200.

Li, Z., X. Xi, M. Gu, R. Feil, R. D. Ye, M. Eigenthaler, F. Hofmann, and X. Du. 2003. "A stimulatory role for cGMP-dependent protein kinase in platelet activation." *Cell* 112 (1):77-86.

Li, Z., G. Zhang, R. Feil, J. Han, and X. Du. 2006. "Sequential activation of p38 and ERK pathways by cGMP-dependent protein kinase leading to activation of the platelet integrin alphaIIb beta3." *Blood* 107 (3):965-72. doi: 10.1182/blood-2005-03-1308.

Li, Z., G. Zhang, J. A. Marjanovic, C. Ruan, and X. Du. 2004. "A platelet secretion pathway mediated by cGMP-dependent protein kinase." *J Biol Chem* 279 (41):42469-75. doi: 10.1074/jbc.M401532200.

Li, Zhenyu, Guoying Zhang, Junling Liu, Aleksandra Stojanovic, Changgeng Ruan, Clifford A. Lowell, and Xiaoping Du. 2010. "An Important Role of the Src Family Kinase Lyn in Stimulating Platelet Granule Secretion." *Journal of Biological Chemistry* 285 (17):12559-12570. doi: 10.1074/jbc.M109.098756.

Liu, G., and I. N. Pessah. 1994. "Molecular interaction between ryanodine receptor and glycoprotein triadin involves redox cycling of functionally important hyperreactive sulfhydryls." *J Biol Chem* 269 (52):33028-34.

Liu, J., T. I. Pestina, M. C. Berndt, C. W. Jackson, and T. K. Gartner. 2005. "Botrocetin/VWF-induced signaling through GPIb-IX-V produces TxA2 in an alphaIIbbeta3- and aggregation-independent manner." *Blood* 106 (8):2750-6. doi: 10.1182/blood-2005-04-1667.

Lopez, J. A. 1994. "The platelet glycoprotein Ib-IX complex." *Blood Coagul Fibrinolysis* 5 (1):97-119.

Lopez, J. A., R. K. Andrews, V. Afshar-Kharghan, and M. C. Berndt. 1998. "Bernard-Soulier syndrome." *Blood* 91 (12):4397-418.

Lopez, J. A., D. W. Chung, K. Fujikawa, F. S. Hagen, E. W. Davie, and G. J. Roth. 1988. "The alpha and beta chains of human platelet glycoprotein Ib are both transmembrane proteins containing a leucine-rich amino acid sequence." *Proc Natl Acad Sci U S A* 85 (7):2135-9.

Lopez, J. A., D. W. Chung, K. Fujikawa, F. S. Hagen, T. Papayannopoulou, and G. J. Roth. 1987. "Cloning of the alpha chain of human platelet glycoprotein Ib: a transmembrane protein with homology to leucine-rich alpha 2-glycoprotein." *Proc Natl Acad Sci U S A* 84 (16):5615-9.

Ma, D., J. Zhang, H. F. Lin, J. Italiano, and R. I. Handin. 2011. "The identification and characterization of zebrafish hematopoietic stem cells." *Blood* 118 (2):289-97. doi: 10.1182/blood-2010-12-327403.

Ma, Y. Q., J. Qin, C. Wu, and E. F. Plow. 2008. "Kindlin-2 (Mig-2): a co-activator of beta3 integrins." *J Cell Biol* 181 (3):439-46. doi: 10.1083/jcb.200710196.

Mahaut-Smith, M. P., S. J. Ennion, M. G. Rolf, and R. J. Evans. 2000. "ADP is not an agonist at P2X(1) receptors: evidence for separate receptors stimulated by ATP and ADP on human platelets." *Br J Pharmacol* 131 (1):108-14. doi: 10.1038/sj.bjp.0703517.

Makni-Maalej, Karama, Mélanie Chiandotto, Margarita Hurtado-Nedelec, Samia Bedouhene, Marie-Anne Gougerot-Pocidalo, Pham My-Chan Dang, and Jamel El-Benna. 2013. "Zymosan induces NADPH oxidase activation in human neutrophils by inducing the phosphorylation of p47phox and the activation of Rac2: Involvement of protein tyrosine kinases, PI3Kinase, PKC, ERK1/2 and p38MAPkinase." *Biochemical Pharmacology* 85 (1):92-100. doi: http://dx.doi.org/10.1016/j.bcp.2012.10.010.

Malinin, N. L., L. Zhang, J. Choi, A. Ciocea, O. Razorenova, Y. Q. Ma, E. A. Podrez, M. Tosi, D. P. Lennon, A. I. Caplan, S. B. Shurin, E. F. Plow, and T. V. Byzova. 2009. "A point mutation in KINDLIN3 ablates activation of three integrin subfamilies in humans." *Nat Med* 15 (3):313-8. doi: 10.1038/nm.1917.

Mangin, P., T. David, V. Lavaud, S. L. Cranmer, I. Pikovski, S. P. Jackson, M. C. Berndt, J. P. Cazenave, C. Gachet, and F. Lanza. 2004. "Identification of a novel 14-3-3zeta binding site within the cytoplasmic tail of platelet glycoprotein Ibalpha." *Blood* 104 (2):420-7. doi: 10.1182/blood-2003-08-2881.

Marchese, P., M. Murata, M. Mazzucato, P. Pradella, L. De Marco, J. Ware, and Z. M. Ruggeri. 1995. "Identification of three tyrosine residues of glycoprotein Ib alpha with distinct roles in von Willebrand factor and alpha-thrombin binding." *J Biol Chem* 270 (16):9571-8.

Menashi, S., C. Davis, and N. Crawford. 1982. "Calcium uptake associated with an intracellular membrane fraction prepared from human blood platelets by high-voltage, free-flow electrophoresis." *FEBS Lett* 140 (2):298-302.

Miyata, S., S. Goto, A. B. Federici, J. Ware, and Z. M. Ruggeri. 1996. "Conformational changes in the A1 domain of von Willebrand factor modulating the interaction with platelet glycoprotein Ibalpha." *J Biol Chem* 271 (15):9046-53.

Moake, J. L. 2002. "Thrombotic thrombocytopenic purpura: the systemic clumping "plague"." *Annu Rev Med* 53:75-88. doi: 10.1146/annurev.med.53.082901.103948.

Montanez, E., S. Ussar, M. Schifferer, M. Bosl, R. Zent, M. Moser, and R. Fassler. 2008. "Kindlin-2 controls bidirectional signaling of integrins." *Genes Dev* 22 (10):1325-30. doi: 10.1101/gad.469408.

Moog, S., P. Mangin, N. Lenain, C. Strassel, C. Ravanat, S. Schuhler, M. Freund, M. Santer, M. Kahn, B. Nieswandt, C. Gachet, J. P. Cazenave, and F. Lanza. 2001. "Platelet glycoprotein V binds to collagen and participates in platelet adhesion and aggregation." *Blood* 98 (4):1038-46.

Morris, C. D., and S. Carson. 2003. "Routine vitamin supplementation to prevent cardiovascular disease: a summary of the evidence for the U.S. Preventive Services Task Force." *Ann Intern Med* 139 (1):56-70.

Moser, M., B. Nieswandt, S. Ussar, M. Pozgajova, and R. Fassler. 2008. "Kindlin-3 is essential for integrin activation and platelet aggregation." *Nat Med* 14 (3):325-30. doi: 10.1038/nm1722.

Munday, A. D., M. C. Berndt, and C. A. Mitchell. 2000. "Phosphoinositide 3-kinase forms a complex with platelet membrane glycoprotein Ib-IX-V complex and 14-3-3zeta." *Blood* 96 (2):577-84.

Murugappa, S., and S. P. Kunapuli. 2006. "The role of ADP receptors in platelet function." *Front Biosci* 11:1977-86.

Nakanishi-Matsui, M., Y. W. Zheng, D. J. Sulciner, E. J. Weiss, M. J. Ludeman, and S. R. Coughlin. 2000. "PAR3 is a cofactor for PAR4 activation by thrombin." *Nature* 404 (6778):609-13. doi: 10.1038/35007085.

Narumiya, S., N. Hirata, T. Namba, Y. Hayashi, F. Ushikubi, Y. Sugimoto, M. Negishi, and A. Ichikawa. 1993. "Structure and function of prostanoid receptors." *J Lipid Mediat* 6 (1-3):155-61.

Nessel, C. O. 2005. "On "the P2 receptors and congenital platelet function defects" (Semin Thromb Hemost 2005;31:168-173)." *Semin Thromb Hemost* 31 (5):610. doi: 10.1055/s-2005-922232.

Nieswandt, B., C. Brakebusch, W. Bergmeier, V. Schulte, D. Bouvard, R. Mokhtari-Nejad, T. Lindhout, J. W. Heemskerk, H. Zirngibl, and R. Fassler. 2001. "Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen." *Embo j* 20 (9):2120-30. doi: 10.1093/emboj/20.9.2120.

Nieswandt, B., and S. P. Watson. 2003. "Platelet-collagen interaction: is GPVI the central receptor?" *Blood* 102 (2):449-61. doi: 10.1182/blood-2002-12-3882.

Nurden, A. T., and J. P. Caen. 1974. "An abnormal platelet glycoprotein pattern in three cases of Glanzmann's thrombasthenia." *Br J Haematol* 28 (2):253-60.

Nystedt, S., K. Emilsson, C. Wahlestedt, and J. Sundelin. 1994. "Molecular cloning of a potential proteinase activated receptor." *Proc Natl Acad Sci U S A* 91 (20):9208-12.

Obergfell, A., K. Eto, A. Mocsai, C. Buensuceso, S. L. Moores, J. S. Brugge, C. A. Lowell, and S. J. Shattil. 2002. "Coordinate interactions of Csk, Src, and Syk kinases with [alpha]IIb[beta]3 initiate integrin signaling to the cytoskeleton." *J Cell Biol* 157 (2):265-75. doi: 10.1083/jcb.200112113.

Odell, T. T., and C. W. Jackson. 1968. "Polyploidy and Maturation of Rat Megakaryocytes." *Blood* 32 (1):102-110.

Odell, T. T., Jr., C. W. Jackson, and T. J. Friday. 1970. "Megakaryocytopoiesis in rats with special reference to polyploidy." *Blood* 35 (6):775-82.

Osler, William. 1886. "On certain problems in the physiology of the blood corpuscles." *The Medical News* 48:421-425.

Oury, C., E. Toth-Zsamboki, C. Thys, J. Tytgat, J. Vermylen, and M. F. Hoylaerts. 2001. "The ATP-gated P2X1 ion channel acts as a positive regulator of platelet responses to collagen." *Thromb Haemost* 86 (5):1264-71.

Pao, M., E. A. Wiggs, M. M. Anastacio, J. Hyun, E. S. DeCarlo, J. T. Miller, V. L. Anderson, H. L. Malech, J. I. Gallin, and S. M. Holland. 2004. "Cognitive function in patients with chronic granulomatous disease: a preliminary report." *Psychosomatics* 45 (3):230-4. doi: 10.1176/appi.psy.45.3.230.

Paravicini, T. M., S. Chrissobolis, G. R. Drummond, and C. G. Sobey. 2004. "Increased NADPH-oxidase activity and Nox4 expression during chronic hypertension is associated with enhanced cerebral vasodilatation to NADPH in vivo." *Stroke* 35 (2):584-9. doi: 10.1161/01.str.0000112974.37028.58.

Paravicini, T. M., A. A. Miller, G. R. Drummond, and C. G. Sobey. 2006. "Flow-induced cerebral vasodilatation in vivo involves activation of phosphatidylinositol-3 kinase, NADPH-oxidase, and nitric oxide synthase." *J Cereb Blood Flow Metab* 26 (6):836-45. doi: 10.1038/sj.jcbfm.9600235.

Parkos, C. A., M. C. Dinauer, A. J. Jesaitis, S. H. Orkin, and J. T. Curnutte. 1989. "Absence of both the 91kD and 22kD subunits of human neutrophil cytochrome b in two genetic forms of chronic granulomatous disease." *Blood* 73 (6):1416-20.

Pasquet, J. M., R. Bobe, B. Gross, M. P. Gratacap, M. G. Tomlinson, B. Payrastre, and S. P. Watson. 1999. "A collagen-related peptide regulates phospholipase Cgamma2 via phosphatidylinositol 3-kinase in human platelets." *Biochem J* 342 (Pt 1):171-7.

Pignatelli, Pasquale, Fabio M. Pulcinelli, Luisa Lenti, Pier Paolo Gazzaniga, and Francesco Violi. 1998. "Hydrogen Peroxide Is Involved in Collagen-Induced Platelet Activation." *Blood* 91 (2):484-490.

Pollock, J. D., D. A. Williams, M. A. Gifford, L. L. Li, X. Du, J. Fisherman, S. H. Orkin, C. M. Doerschuk, and M. C. Dinauer. 1995. "Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production." *Nat Genet* 9 (2):202-9. doi: 10.1038/ng0295-202.

Quek, L. S., J. Bolen, and S. P. Watson. 1998. "A role for Bruton's tyrosine kinase (Btk) in platelet activation by collagen." *Curr Biol* 8 (20):1137-40.

Quek, L. S., J. M. Pasquet, I. Hers, R. Cornall, G. Knight, M. Barnes, M. L. Hibbs, A. R. Dunn, C. A. Lowell, and S. P. Watson. 2000. "Fyn and Lyn phosphorylate the Fc receptor gamma chain downstream of glycoprotein VI in murine platelets, and Lyn regulates a novel feedback pathway." *Blood* 96 (13):4246-53.

Ramakrishnan, V., F. DeGuzman, M. Bao, S. W. Hall, L. L. Leung, and D. R. Phillips. 2001. "A thrombin receptor function for platelet glycoprotein Ib-IX unmasked by cleavage of glycoprotein V." *Proc Natl Acad Sci U S A* 98 (4):1823-8. doi: 10.1073/pnas.98.4.1823.

Rasmussen, U. B., V. Vouret-Craviari, S. Jallat, Y. Schlesinger, G. Pages, A. Pavirani, J. P. Lecocq, J. Pouyssegur, and E. Van Obberghen-Schilling. 1991. "cDNA cloning and expression of a hamster alpha-thrombin receptor coupled to Ca2+ mobilization." *FEBS Lett* 288 (1-2):123-8.

Redondo, P. C., G. M. Salido, J. A. Rosado, and J. A. Pariente. 2004. "Effect of hydrogen peroxide on Ca2+ mobilisation in human platelets through sulphydryl oxidation dependent and independent mechanisms." *Biochem Pharmacol* 67 (3):491-502. doi: 10.1016/j.bcp.2003.09.031.

Richardson, J. L., R. A. Shivdasani, C. Boers, J. H. Hartwig, and J. E. Italiano, Jr. 2005. "Mechanisms of organelle transport and capture along proplatelets during platelet production." *Blood* 106 (13):4066-75. doi: 10.1182/blood-2005-06-2206.

Robblee, L. S., D. Shepro, and F. A. Belamarich. 1973. "Calcium uptake and associated adenosine triphosphatase activity of isolated platelet membranes." *J Gen Physiol* 61 (4):462-81.

Rolf, M. G., C. A. Brearley, and M. P. Mahaut-Smith. 2001. "Platelet shape change evoked by selective activation of P2X1 purinoceptors with alpha,beta-methylene ATP." *Thromb Haemost* 85 (2):303-8.

Rolf, M. G., and M. P. Mahaut-Smith. 2002. "Effects of enhanced P2X1 receptor Ca2+ influx on functional responses in human platelets." *Thromb Haemost* 88 (3):495-502. doi: 10.1267/thro88030495.

Rosenberg, S., A. Stracher, and K. Burridge. 1981. "Isolation and characterization of a calcium-sensitive alpha-actinin-like protein from human platelet cytoskeletons." *J Biol Chem* 256 (24):12986-91.

Ruggeri, Z. M. 1997. "von Willebrand factor." *J Clin Invest* 99 (4):559-64. doi: 10.1172/jci119195.

Russo, A., U. J. Soh, and J. Trejo. 2009. "Proteases display biased agonism at protease-activated receptors: location matters!" *Mol Interv* 9 (2):87-96. doi: 10.1124/mi.9.2.8.

Sage, S. O., and T. J. Rink. 1987. "The kinetics of changes in intracellular calcium concentration in fura-2-loaded human platelets." *J Biol Chem* 262 (34):16364-9.

Savi, P., P. Beauverger, C. Labouret, M. Delfaud, V. Salel, M. Kaghad, and J. M. Herbert. 1998. "Role of P2Y1 purinoceptor in ADP-induced platelet activation." *FEBS Lett* 422 (3):291-5.

Schiffrin, E. L. 2010. "Antioxidants in hypertension and cardiovascular disease." *Mol Interv* 10 (6):354-62. doi: 10.1124/mi.10.6.4.

Schorer, A. E., C. F. Moldow, and M. E. Rick. 1987. "Interleukin 1 or endotoxin increases the release of von Willebrand factor from human endothelial cells." *Br J Haematol* 67 (2):193-7.

Schwer, H. D., P. Lecine, S. Tiwari, J. E. Italiano, Jr., J. H. Hartwig, and R. A. Shivdasani. 2001. "A lineage-restricted and divergent beta-tubulin isoform is essential for the biogenesis, structure and function of blood platelets." *Curr Biol* 11 (8):579-86.

Seitz, I., S. Hess, H. Schulz, R. Eckl, G. Busch, H. P. Montens, R. Brandl, S. Seidl, A. Schomig, and I. Ott. 2007. "Membrane-type serine protease-1/matriptase induces interleukin-6 and -8 in endothelial cells by activation of protease-activated receptor-2: potential implications in atherosclerosis." *Arterioscler Thromb Vasc Biol* 27 (4):769-75. doi: 10.1161/01.atv.0000258862.61067.14.

Senis, Y. A., M. G. Tomlinson, S. Ellison, A. Mazharian, J. Lim, Y. Zhao, K. N. Kornerup, J. M. Auger, S. G. Thomas, T. Dhanjal, N. Kalia, J. W. Zhu, A. Weiss, and S. P. Watson. 2009. "The tyrosine phosphatase CD148 is an essential positive regulator of platelet activation and thrombosis." *Blood* 113 (20):4942-54. doi: 10.1182/blood-2008-08-174318.

Shattil, S. J., and P. J. Newman. 2004. "Integrins: dynamic scaffolds for adhesion and signaling in platelets." *Blood* 104 (6):1606-15. doi: 10.1182/blood-2004-04-1257.

Shattil, Sanford J., Chungho Kim, and Mark H. Ginsberg. 2010. "The final steps of integrin activation: the end game." *Nat Rev Mol Cell Biol* 11 (4):288-300.

Shen, B., X. Zhao, K. A. O'Brien, A. Stojanovic-Terpo, M. K. Delaney, K. Kim, J. Cho, S. C. Lam, and X. Du. 2013. "A directional switch of integrin signalling and a new anti-thrombotic strategy." *Nature* 503 (7474):131-5. doi: 10.1038/nature12613.

Shen, Y., J. F. Dong Jf, G. M. Romo, W. Arceneaux, A. Aprico, E. E. Gardiner, J. A. Lopez, M. C. Berndt, and R. K. Andrews. 2002. "Functional analysis of the C-terminal flanking sequence of platelet glycoprotein Ib alpha using canine-human chimeras." *Blood* 99 (1):145-50.

Shen, Y., G. M. Romo, J. F. Dong, A. Schade, L. V. McIntire, D. Kenny, J. C. Whisstock, M. C. Berndt, J. A. Lopez, and R. K. Andrews. 2000. "Requirement of leucine-rich repeats of glycoprotein (GP) Ibalpha for shear-dependent and static binding of von Willebrand factor to the platelet membrane GP Ib-IX-V complex." *Blood* 95 (3):903-10.

Shrimpton, C. N., G. Borthakur, S. Larrucea, M. A. Cruz, J. F. Dong, and J. A. Lopez. 2002. "Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation." *J Exp Med* 196 (8):1057-66.

Siedlecki, C. A., B. J. Lestini, K. K. Kottke-Marchant, S. J. Eppell, D. L. Wilson, and R. E. Marchant. 1996. "Shear-dependent changes in the three-dimensional structure of human von Willebrand factor." *Blood* 88 (8):2939-50.

Simon, D. I., Z. Chen, H. Xu, C. Q. Li, Jf Dong, L. V. McIntire, C. M. Ballantyne, L. Zhang, M. I. Furman, M. C. Berndt, and J. A. Lopez. 2000. "Platelet glycoprotein ibalpha is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18)." *J Exp Med* 192 (2):193-204.

Sixma, J. J., J. W. Slot, and H. J. Geuze. 1989. "Immunocytochemical localization of platelet granule proteins." *Methods Enzymol* 169:301-11.

Stojanovic, A., J. A. Marjanovic, V. M. Brovkovych, X. Peng, N. Hay, R. A. Skidgel, and X. Du. 2006. "A phosphoinositide 3-kinase-AKT-nitric oxide-cGMP signaling pathway in stimulating platelet secretion and aggregation." *J Biol Chem* 281 (24):16333-9. doi: 10.1074/jbc.M512378200.

Sugamura, Koichi, and Jr John F. Keaney. 2011. "Reactive oxygen species in cardiovascular disease." *Free Radical Biology and Medicine* 51 (5):978-992. doi: http://dx.doi.org/10.1016/j.freeradbiomed.2011.05.004.

Sugiyama, T., M. Okuma, F. Ushikubi, S. Sensaki, K. Kanaji, and H. Uchino. 1987. "A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia." *Blood* 69 (6):1712-20.

Suh, Y. A., R. S. Arnold, B. Lassegue, J. Shi, X. Xu, D. Sorescu, A. B. Chung, K. K. Griendling, and J. D. Lambeth. 1999. "Cell transformation by the superoxide-generating oxidase Mox1." *Nature* 401 (6748):79-82. doi: 10.1038/43459.

Suzuki-Inoue, K., D. Tulasne, Y. Shen, T. Bori-Sanz, O. Inoue, S. M. Jung, M. Moroi, R. K. Andrews, M. C. Berndt, and S. P. Watson. 2002. "Association of Fyn and Lyn with the proline-rich domain of glycoprotein VI regulates intracellular signaling." *J Biol Chem* 277 (24):21561-6. doi: 10.1074/jbc.M201012200.

Svensson, J., M. Hamberg, and B. Samuelsson. 1976. "On the formation and effects of thromboxane A2 in human platelets." *Acta Physiol Scand* 98 (3):285-94. doi: 10.1111/j.1748-1716.1976.tb10313.x.

Szanto, I., L. Rubbia-Brandt, P. Kiss, K. Steger, B. Banfi, E. Kovari, F. Herrmann, A. Hadengue, and K. H. Krause. 2005. "Expression of NOX1, a superoxide-generating NADPH oxidase, in colon cancer and inflammatory bowel disease." *J Pathol* 207 (2):164-76. doi: 10.1002/path.1824.

Tadokoro, S., S. J. Shattil, K. Eto, V. Tai, R. C. Liddington, J. M. de Pereda, M. H. Ginsberg, and D. A. Calderwood. 2003. "Talin binding to integrin beta tails: a final common step in integrin activation." *Science* 302 (5642):103-6. doi: 10.1126/science.1086652.

Takahara, K., R. Murray, G. A. FitzGerald, and D. J. Fitzgerald. 1990. "The response to thromboxane A2 analogues in human platelets. Discrimination of two binding sites linked to distinct effector systems." *J Biol Chem* 265 (12):6836-44.

Takano, S., J. Kimura, I. Matsuoka, and T. Ono. 1999. "No requirement of P2X1 purinoceptors for platelet aggregation." *Eur J Pharmacol* 372 (3):305-9.

Toth-Zsamboki, E., C. Oury, H. Cornelissen, R. De Vos, J. Vermylen, and M. F. Hoylaerts. 2003. "P2X1-mediated ERK2 activation amplifies the collagen-induced platelet secretion by enhancing myosin light chain kinase activation." *J Biol Chem* 278 (47):46661-7. doi: 10.1074/jbc.M308452200.

Ueno, N., R. Takeya, K. Miyano, H. Kikuchi, and H. Sumimoto. 2005. "The NADPH oxidase Nox3 constitutively produces superoxide in a p22phox-dependent manner: its regulation by oxidase organizers and activators." *J Biol Chem* 280 (24):23328-39. doi: 10.1074/jbc.M414548200.

Ueyama, T., M. Geiszt, and T. L. Leto. 2006. "Involvement of Rac1 in activation of multicomponent Nox1- and Nox3-based NADPH oxidases." *Mol Cell Biol* 26 (6):2160-74. doi: 10.1128/mcb.26.6.2160-2174.2006.

Ulsemer, P., F. Lanza, M. J. Baas, A. Schwartz, C. Ravanat, M. E. Briquel, S. Cranmer, S. Jackson, J. P. Cazenave, and C. de la Salle. 2000. "Role of the leucine-rich domain of platelet GPIbalpha in correct post-translational processing--the Nancy I Bernard-Soulier mutation expressed on CHO cells." *Thromb Haemost* 84 (1):104-11.

Vivekananthan, D. P., M. S. Penn, S. K. Sapp, A. Hsu, and E. J. Topol. 2003. "Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials." *Lancet* 361 (9374):2017-23. doi: 10.1016/s0140-6736(03)13637-9.

Vlahos, C. J., W. F. Matter, R. F. Brown, A. E. Traynor-Kaplan, P. G. Heyworth, E. R. Prossnitz, R. D. Ye, P. Marder, J. A. Schelm, K. J. Rothfuss, and et al. 1995. "Investigation of neutrophil signal transduction using a specific inhibitor of phosphatidylinositol 3-kinase." *J Immunol* 154 (5):2413-22.

Vu, T. K., D. T. Hung, V. I. Wheaton, and S. R. Coughlin. 1991. "Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation." *Cell* 64 (6):1057-68.

Wagner, C. L., M. A. Mascelli, D. S. Neblock, H. F. Weisman, B. S. Coller, and R. E. Jordan. 1996. "Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets." *Blood* 88 (3):907-14.

Walker, T. R., and S. P. Watson. 1993. "Synergy between Ca2+ and protein kinase C is the major factor in determining the level of secretion from human platelets." *Biochem J* 289 (Pt 1):277-82.

Wang, G., J. Anrather, J. Huang, R. C. Speth, V. M. Pickel, and C. Iadecola. 2004. "NADPH oxidase contributes to angiotensin II signaling in the nucleus tractus solitarius." *J Neurosci* 24 (24):5516-24. doi: 10.1523/jneurosci.1176-04.2004.

Wang, L., O. Ostberg, A. K. Wihlborg, H. Brogren, S. Jern, and D. Erlinge. 2003. "Quantification of ADP and ATP receptor expression in human platelets." *J Thromb Haemost* 1 (2):330-6.

Wang, X., S. Takeda, S. Mochizuki, R. Jindal, and N. S. Dhalla. 1999. "Mechanisms of Hydrogen Peroxide-Induced Increase in Intracellular Calcium in Cardiomyocytes." *J Cardiovasc Pharmacol Ther* 4 (1):41-48. doi: 10.1053/jcpt00400041.

Ward, C. M., R. K. Andrews, A. I. Smith, and M. C. Berndt. 1996. "Mocarhagin, a novel cobra venom metalloproteinase, cleaves the platelet von Willebrand factor receptor glycoprotein Ibalpha. Identification of the sulfated tyrosine/anionic sequence Tyr-276-Glu-282 of glycoprotein Ibalpha as a binding site for von Willebrand factor and alpha-thrombin." *Biochemistry* 35 (15):4929-38. doi: 10.1021/bi952456c.

Wardell, M. R., C. C. Reynolds, M. C. Berndt, R. W. Wallace, and J. E. Fox. 1989. "Platelet glycoprotein Ib beta is phosphorylated on serine 166 by cyclic AMP-dependent protein kinase." *J Biol Chem* 264 (26):15656-61.

Ware, J. 1998. "Molecular analyses of the platelet glycoprotein Ib-IX-V receptor." *Thromb Haemost* 79 (3):466-78.

Ware, J., A. Corken, and R. Khetpal. 2013. "Platelet function beyond hemostasis and thrombosis." *Curr Opin Hematol* 20 (5):451-6. doi: 10.1097/MOH.0b013e32836344d3.

Watanabe, N., H. Nakajima, H. Suzuki, A. Oda, Y. Matsubara, M. Moroi, Y. Terauchi, T. Kadowaki, H. Suzuki, S. Koyasu, Y. Ikeda, and M. Handa. 2003. "Functional phenotype of phosphoinositide 3-kinase p85alpha-null platelets characterized by an impaired response to GP VI stimulation." *Blood* 102 (2):541-8. doi: 10.1182/blood-2002-11-3327.

Watson, S. P., N. Asazuma, B. Atkinson, O. Berlanga, D. Best, R. Bobe, G. Jarvis, S. Marshall, D. Snell, M. Stafford, D. Tulasne, J. Wilde, P. Wonerow, and J. Frampton. 2001. "The role of ITAM- and ITIM-coupled receptors in platelet activation by collagen." *Thromb Haemost* 86 (1):276-88.

Watson, S. P., J. M. Auger, O. J. McCarty, and A. C. Pearce. 2005. "GPVI and integrin alphaIIb beta3 signaling in platelets." *J Thromb Haemost* 3 (8):1752-62. doi: 10.1111/j.1538-7836.2005.01429.x.

Wentworth, P., Jr., J. E. McDunn, A. D. Wentworth, C. Takeuchi, J. Nieva, T. Jones, C. Bautista, J. M. Ruedi, A. Gutierrez, K. D. Janda, B. M. Babior, A. Eschenmoser, and R. A. Lerner. 2002. "Evidence for antibody-catalyzed ozone formation in bacterial killing and inflammation." *Science* 298 (5601):2195-9. doi: 10.1126/science.1077642.

Whisstock, J. C., Y. Shen, J. A. Lopez, R. K. Andrews, and M. C. Berndt. 2002. "Molecular modeling of the seven tandem leucine-rich repeats within the ligand-binding region of platelet glycoprotein Ib alpha." *Thromb Haemost* 87 (2):329-33.

White, J. G. 1968. "Effects of colchicine and Vinca alkaloids on human platelets. I. Influence on platelet microtubules and contractile function." *The American Journal of Pathology* 53 (2):281-291.

White, J. G. 1972. "Uptake of latex particles by blood platelets: phagocytosis or sequestration?" *Am J Pathol* 69 (3):439-58.

White, J. G. 1988. "Platelet membrane ultrastructure and its changes during platelet activation." *Prog Clin Biol Res* 283:1-32.

White, J. G., and W. Krivit. 1967. "An ultrastructural basis for the shape changes induced in platelets by chilling." *Blood* 30 (5):625-35.

Woodside, D. G., A. Obergfell, L. Leng, J. L. Wilsbacher, C. K. Miranti, J. S. Brugge, S. J. Shattil, and M. H. Ginsberg. 2001. "Activation of Syk protein tyrosine kinase through interaction with integrin beta cytoplasmic domains." *Curr Biol* 11 (22):1799-804.

Woulfe, D., H. Jiang, A. Morgans, R. Monks, M. Birnbaum, and L. F. Brass. 2004. "Defects in secretion, aggregation, and thrombus formation in platelets from mice lacking Akt2." *J Clin Invest* 113 (3):441-50. doi: 10.1172/jci20267.

Wu, D., M. Meiring, H. F. Kotze, H. Deckmyn, and N. Cauwenberghs. 2002. "Inhibition of platelet glycoprotein Ib, glycoprotein IIb/IIIa, or both by monoclonal antibodies prevents arterial thrombosis in baboons." *Arterioscler Thromb Vasc Biol* 22 (2):323-8.

Wu, Y., N. Asazuma, K. Satoh, Y. Yatomi, T. Takafuta, M. C. Berndt, and Y. Ozaki. 2003. "Interaction between von Willebrand factor and glycoprotein Ib activates Src kinase in human platelets: role of phosphoinositide 3-kinase." *Blood* 101 (9):3469-76. doi: 10.1182/blood-2002-03-0806.

Xi, X., R. J. Bodnar, Z. Li, S. C. Lam, and X. Du. 2003. "Critical roles for the COOH-terminal NITY and RGT sequences of the integrin beta3 cytoplasmic domain in inside-out and outside-in signaling." *J Cell Biol* 162 (2):329-39. doi: 10.1083/jcb.200303120.

Xi, X., P. Flevaris, A. Stojanovic, A. Chishti, D. R. Phillips, S. C. Lam, and X. Du. 2006. "Tyrosine phosphorylation of the integrin beta 3 subunit regulates beta 3 cleavage by calpain." *J Biol Chem* 281 (40):29426-30. doi: 10.1074/jbc.C600039200.

Xiao, B., S. J. Smerdon, D. H. Jones, G. G. Dodson, Y. Soneji, A. Aitken, and S. J. Gamblin. 1995. "Structure of a 14-3-3 protein and implications for coordination of multiple signalling pathways." *Nature* 376 (6536):188-91. doi: 10.1038/376188a0.

Xu, W. F., H. Andersen, T. E. Whitmore, S. R. Presnell, D. P. Yee, A. Ching, T. Gilbert, E. W. Davie, and D. C. Foster. 1998. "Cloning and characterization of human protease-activated receptor 4." *Proc Natl Acad Sci U S A* 95 (12):6642-6.

Yanaga, F., A. Poole, J. Asselin, R. Blake, G. L. Schieven, E. A. Clark, C. L. Law, and S. P. Watson. 1995. "Syk interacts with tyrosine-phosphorylated proteins in human platelets activated by collagen and cross-linking of the Fc gamma-IIA receptor." *Biochem J* 311 (Pt 2):471-8.

Yap, C. L., K. E. Anderson, S. C. Hughan, S. M. Dopheide, H. H. Salem, and S. P. Jackson. 2002. "Essential role for phosphoinositide 3-kinase in shear-dependent signaling between platelet glycoprotein Ib/V/IX and integrin alpha(IIb)beta(3)." *Blood* 99 (1):151-8.

Ye, F., B. G. Petrich, P. Anekal, C. T. Lefort, A. Kasirer-Friede, S. J. Shattil, R. Ruppert, M. Moser, R. Fassler, and M. H. Ginsberg. 2013. "The mechanism of kindlin-mediated activation of integrin alphaIIbbeta3." *Curr Biol* 23 (22):2288-95. doi: 10.1016/j.cub.2013.09.050.

Yi, X. Y., V. X. Li, F. Zhang, F. Yi, D. R. Matson, M. T. Jiang, and P. L. Li. 2006. "Characteristics and actions of NAD(P)H oxidase on the sarcoplasmic reticulum of coronary artery smooth muscle." *Am J Physiol Heart Circ Physiol* 290 (3):H1136-44. doi: 10.1152/ajpheart.00296.2005.

Yin, H., A. Stojanovic, N. Hay, and X. Du. 2008. "The role of Akt in the signaling pathway of the glycoprotein Ib-IX induced platelet activation." *Blood* 111 (2):658-65. doi: 10.1182/blood-2007-04-085514.

Zhang, G., B. Xiang, S. Ye, M. Chrzanowska-Wodnicka, A. J. Morris, T. K. Gartner, S. W. Whiteheart, G. C. White, 2nd, S. S. Smyth, and Z. Li. 2011. "Distinct roles for Rap1b protein in platelet secretion and integrin alphaIIbbeta3 outside-in signaling." *J Biol Chem* 286 (45):39466-77. doi: 10.1074/jbc.M111.239608.

VI. APPENDIX

WOLTERS KLUWER HEALTH, INC. LICENSE TERMS AND CONDITIONS

Jun 20, 2016

This Agreement between Zheng Xu ("You") and Wolters Kluwer Health, Inc. ("Wolters Kluwer Health, Inc.") consists of your license details and the terms and conditions provided by Wolters Kluwer Health, Inc. and Copyright Clearance Center.

License Number	3893180231818
License date	Jun 20, 2016
Licensed Content Publisher	Wolters Kluwer Health, Inc.
Licensed Content Publication	Current Opinion in Hematology
Licensed Content Title	Signaling and regulation of the platelet glycoprotein Ib–IX–V complex
Licensed Content Author	Xiaoping Du
Licensed Content Date	Jan 1, 2007
Licensed Content Volume Number	14
Licensed Content Issue Number	3
Type of Use	Dissertation/Thesis
Requestor type	Individual
Portion	Figures/table/illustration
Number of figures/tables/illustrations	1
Figures/tables/illustrations used	2
Author of this Wolters Kluwer article	No
Title of your thesis / dissertation	Integrin alphaIIbbeta3-dependent Reactive Oxygen Species Production in Platelets
Expected completion date	Jul 2016
Estimated size(pages)	100
Requestor Location	Zheng Xu 1309 W Flournoy St Apt 2 CHICAGO, IL 60607 United States Attn: Zheng Xu

Publisher Tax ID	13-2932696
Billing Type	Invoice
Billing Address	Zheng Xu 1309 W Flournoy St Apt 2 CHICAGO, IL 60607 United States Attn: Zheng Xu
Total	0.00 USD

Terms and Conditions

Wolters Kluwer Terms and Conditions

1. **Transfer of License:** Wolters Kluwer hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions herein.
2. **Credit Line:** will be prominently placed and include: For books – the author(s), title of book, editor, copyright holder, year of publication; For journals – the author(s), title of article, title of journal, volume number, issue number and inclusive pages.
3. **Warranties:** The requestor warrants that the material shall not be used in any manner which may be considered derogatory to the title, content, or authors of the material, or to Wolters Kluwer.
4. **Indemnity:** You hereby indemnify and hold harmless Wolters Kluwer and their respective officers, directors, employees and agents, from and against any and all claims, costs, proceeding or demands arising out of your unauthorized use of the Licensed Material.
5. **Geographical Scope:** Permission granted is non-exclusive, and is valid throughout the world in the English language and the languages specified in your original request.
6. Wolters Kluwer cannot supply the requestor with the original artwork, electronic files or a "clean copy."
7. Permission is valid if the borrowed material is original to a Wolters Kluwer imprint (Lippincott-Raven Publishers, Williams & Wilkins, Lea & Febiger, Harwal, Rapid Science, Little Brown & Company, Harper & Row Medical, American Journal of Nursing Co, and Urban & Schwarzenberg - English Language, Raven Press, Paul Hoeber, Springhouse, Ovid).
8. **Termination of contract:** If you opt not to use the material requested above please notify RightsLink or Wolters Kluwer within 90 days of the original invoice date.
9. This permission does not apply to images that are credited to publications other than Wolters Kluwer books/journals or its Societies. For images credited to non-Wolters Kluwer books or journals, you will need to obtain permission from the source referenced in the figure or table legend or credit line before making any use of the image(s) or table(s).
10. **Modifications:** With the exception of text size or color, no Wolters Kluwer material is permitted to be modified or adapted without publisher approval.
11. **Third party material:** Adaptations are protected by copyright, so if you would like to reuse material that we have adapted from another source, you will need not only our permission, but the permission of the rights holder of the original material. Similarly, if you want to reuse an adaptation of original LWW content that appears in another publishers work, you will need our permission and that of the next publisher. The adaptation should be credited as follows: Adapted with permission from Wolters Kluwer: Book author, title, year of publication or Journal name, article author, title, reference

citation, year of publication. Modifications are permitted on an occasional basis only and permission must be sought by Wolters Kluwer.

12. **Duration of the license:** Permission is granted for a one-time use only within 12 months from the date of this invoice. Rights herein do not apply to future reproductions, editors, revisions, or other derivative works. Once the 12 - month term has expired, permission to renew must be submitted in writing.
    i. For content reused in another journal or book, in print or electronic format, the license is one-time use and lasts for the 1st edition of a book or for the life of the edition in case of journals.
    ii. If your Permission Request is for use on a website (which is not a journal or a book), internet, intranet, or any publicly accessible site, you agree to remove the material from such site after 12 months or else renew your permission request.
13. **Contingent on payment:** While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
14. **Waived permission fee:** If the permission fee for the requested use of our material has been waived in this instance, please be advised that your future requests for Wolters Kluwer materials may incur a fee.
15. **Service Description for Content Services:** Subject to these terms of use, any terms set forth on the particular order, and payment of the applicable fee, you may make the following uses of the ordered materials:
    i. **Content Rental:** You may access and view a single electronic copy of the materials ordered for the time period designated at the time the order is placed. Access to the materials will be provided through a dedicated content viewer or other portal, and access will be discontinued upon expiration of the designated time period. An order for Content Rental does not include any rights to print, download, save, create additional copies, to distribute or to reuse in any way the full text or parts of the materials.
    ii. **Content Purchase:** You may access and download a single electronic copy of the materials ordered. Copies will be provided by email or by such other means as publisher may make available from time to time. An order for Content Purchase does not include any rights to create additional copies or to distribute copies of the materials.

For Journals Only:

1. Please note that articles in the **ahead-of-print stage** of publication can be cited and the content may be re-used by including the date of access and the unique DOI number. Any final changes in manuscripts will be made at the time of print publication and will be reflected in the final electronic version of the issue. Disclaimer: Articles appearing in the Published Ahead-of-Print section have been peer-reviewed and accepted for publication in the relevant journal and posted online before print publication. Articles appearing as publish ahead-of-print may contain statements, opinions, and information that have errors in facts, figures, or interpretation. Accordingly, Wolters Kluwer, the editors and authors and their respective employees are not responsible or liable for the use of any

such inaccurate or misleading data, opinion or information contained in the articles in this section.

2. Where a journal is being published by a learned society, the details of that society must be included in the credit line.
    i. **For Open Access journals:** The following statement needs to be added when reprinting the material in Open Access journals only: "promotional and commercial use of the material in print, digital or mobile device format is prohibited without the permission from the publisher Wolters Kluwer. Please contacthealthpermissions@wolterskluwer.com for further information."
    ii. **Exceptions:** In case of **Disease Colon Rectum, Plastic Reconstructive Surgery, The Green Journal, Critical Care Medicine, Pediatric Critical Care Medicine, the American Heart Publications, the American Academy of Neurology** the following guideline applies: no drug/ trade name or logo can be included in the same page as the material re-used. .
3. **Translations:** If granted permissions to republish a full text article in another language, Wolters Kluwer should be sent a copy of the translated PDF. Please include disclaimer below on all translated copies:
    i. ***Wolters Kluwer and its Societies take no responsibility for the accuracy of the translation from the published English original and are not liable for any errors which may occur.***
4. **Full Text Articles:** Reuse of full text articles in English is prohibited.

STM Signatories Only:

1. Any permission granted for a particular edition will apply also to subsequent editions and for editions in other languages, provided such editions are for the work as a whole in situ and does not involve the separate exploitation of the permitted illustrations or excerpts. Please click here to view the STM guidelines.

Other Terms and Conditions:

v1.3

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

WOLTERS KLUWER HEALTH, INC. LICENSE TERMS AND CONDITIONS

Jun 20, 2016

This Agreement between Zheng Xu ("You") and Wolters Kluwer Health, Inc. ("Wolters Kluwer Health, Inc.") consists of your license details and the terms and conditions provided by Wolters Kluwer Health, Inc. and Copyright Clearance Center.

License Number	3893200241613
License date	Jun 20, 2016
Licensed Content Publisher	Wolters Kluwer Health, Inc.
Licensed Content Publication	ATVB
Licensed Content Title	Signaling During Platelet Adhesion and Activation
Licensed Content Author	Zhenyu Li, M. Keegan Delaney, Kelly A. O'Brien, Xiaoping Du
Licensed Content Date	Dec 1, 2010
Licensed Content Volume Number	30
Licensed Content Issue Number	12
Type of Use	Dissertation/Thesis
Requestor type	Individual
Portion	Figures/table/illustration
Number of figures/tables/illustrations	2
Figures/tables/illustrations used	1, 2
Author of this Wolters Kluwer article	No
Title of your thesis / dissertation	Integrin alphaIIbbeta3-dependent Reactive Oxygen Species Production in Platelets
Expected completion date	Jul 2016
Estimated size(pages)	100
Requestor Location	Zheng Xu 1309 W Flournoy St Apt 2 CHICAGO, IL 60607 United States Attn: Zheng Xu

Publisher Tax ID	13-2932696
Billing Type	Invoice
Billing Address	Zheng Xu 1309 W Flournoy St Apt 2 CHICAGO, IL 60607 United States Attn: Zheng Xu
Total	0.00 USD

Terms and Conditions

Wolters Kluwer Terms and Conditions

1. **Transfer of License:** Wolters Kluwer hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions herein.
2. **Credit Line:** will be prominently placed and include: For books – the author(s), title of book, editor, copyright holder, year of publication; For journals – the author(s), title of article, title of journal, volume number, issue number and inclusive pages.
3. **Warranties:** The requestor warrants that the material shall not be used in any manner which may be considered derogatory to the title, content, or authors of the material, or to Wolters Kluwer.
4. **Indemnity:** You hereby indemnify and hold harmless Wolters Kluwer and their respective officers, directors, employees and agents, from and against any and all claims, costs, proceeding or demands arising out of your unauthorized use of the Licensed Material.
5. **Geographical Scope:** Permission granted is non-exclusive, and is valid throughout the world in the English language and the languages specified in your original request.
6. Wolters Kluwer cannot supply the requestor with the original artwork, electronic files or a "clean copy."
7. Permission is valid if the borrowed material is original to a Wolters Kluwer imprint (Lippincott-Raven Publishers, Williams & Wilkins, Lea & Febiger, Harwal, Rapid Science, Little Brown & Company, Harper & Row Medical, American Journal of Nursing Co, and Urban & Schwarzenberg - English Language, Raven Press, Paul Hoeber, Springhouse, Ovid).
8. **Termination of contract:** If you opt not to use the material requested above please notify RightsLink or Wolters Kluwer within 90 days of the original invoice date.
9. This permission does not apply to images that are credited to publications other than Wolters Kluwer books/journals or its Societies. For images credited to non-Wolters Kluwer books or journals, you will need to obtain permission from the source referenced in the figure or table legend or credit line before making any use of the image(s) or table(s).
10. **Modifications:** With the exception of text size or color, no Wolters Kluwer material is permitted to be modified or adapted without publisher approval.
11. **Third party material:** Adaptations are protected by copyright, so if you would like to reuse material that we have adapted from another source, you will need not only our permission, but the permission of the rights holder of the original material. Similarly, if you want to reuse an adaptation of original LWW content that appears in another publishers work, you will need our permission and that of the next publisher. The adaptation should be credited as follows: Adapted with permission from Wolters

Kluwer: Book author, title, year of publication or Journal name, article author, title, reference citation, year of publication. Modifications are permitted on an occasional basis only and permission must be sought by Wolters Kluwer.

12. **Duration of the license:** Permission is granted for a one-time use only within 12 months from the date of this invoice. Rights herein do not apply to future reproductions, editors, revisions, or other derivative works. Once the 12 - month term has expired, permission to renew must be submitted in writing.
    i. For content reused in another journal or book, in print or electronic format, the license is one-time use and lasts for the 1st edition of a book or for the life of the edition in case of journals.
    ii. If your Permission Request is for use on a website (which is not a journal or a book), internet, intranet, or any publicly accessible site, you agree to remove the material from such site after 12 months or else renew your permission request.
13. **Contingent on payment:** While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
14. **Waived permission fee:** If the permission fee for the requested use of our material has been waived in this instance, please be advised that your future requests for Wolters Kluwer materials may incur a fee.
15. **Service Description for Content Services:** Subject to these terms of use, any terms set forth on the particular order, and payment of the applicable fee, you may make the following uses of the ordered materials:
    i. **Content Rental:** You may access and view a single electronic copy of the materials ordered for the time period designated at the time the order is placed. Access to the materials will be provided through a dedicated content viewer or other portal, and access will be discontinued upon expiration of the designated time period. An order for Content Rental does not include any rights to print, download, save, create additional copies, to distribute or to reuse in any way the full text or parts of the materials.
    ii. **Content Purchase:** You may access and download a single electronic copy of the materials ordered. Copies will be provided by email or by such other means as publisher may make available from time to time. An order for Content Purchase does not include any rights to create additional copies or to distribute copies of the materials.

For Journals Only:

1. Please note that articles in the **ahead-of-print stage** of publication can be cited and the content may be re-used by including the date of access and the unique DOI number. Any final changes in manuscripts will be made at the time of print publication and will be reflected in the final electronic version of the issue. Disclaimer: Articles appearing in the Published Ahead-of-Print section have been peer-reviewed and accepted for publication in the relevant journal and posted online before print publication. Articles appearing as publish ahead-of-print may contain statements,

opinions, and information that have errors in facts, figures, or interpretation. Accordingly, Wolters Kluwer, the editors and authors and their respective employees are not responsible or liable for the use of any such inaccurate or misleading data, opinion or information contained in the articles in this section.

2. Where a journal is being published by a learned society, the details of that society must be included in the credit line.
    i. **For Open Access journals:** The following statement needs to be added when reprinting the material in Open Access journals only: "promotional and commercial use of the material in print, digital or mobile device format is prohibited without the permission from the publisher Wolters Kluwer. Please contacthealthpermissions@wolterskluwer.com for further information."
    ii. **Exceptions:** In case of **Disease Colon Rectum, Plastic Reconstructive Surgery, The Green Journal, Critical Care Medicine, Pediatric Critical Care Medicine, the American Heart Publications, the American Academy of Neurology** the following guideline applies: no drug/ trade name or logo can be included in the same page as the material re-used. .
3. **Translations:** If granted permissions to republish a full text article in another language, Wolters Kluwer should be sent a copy of the translated PDF. Please include disclaimer below on all translated copies:
    i. ***Wolters Kluwer and its Societies take no responsibility for the accuracy of the translation from the published English original and are not liable for any errors which may occur.***
4. **Full Text Articles:** Reuse of full text articles in English is prohibited.

STM Signatories Only:

1. Any permission granted for a particular edition will apply also to subsequent editions and for editions in other languages, provided such editions are for the work as a whole in situ and does not involve the separate exploitation of the permitted illustrations or excerpts. Please click here to view the STM guidelines.

Other Terms and Conditions:

v1.3

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

UNIVERSITY OF ILLINOIS
AT CHICAGO

Office for the Protection of Research Subjects (OPRS)
Office of the Vice Chancellor for Research (MC 672)
203 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Approval Notice

Continuing Review

December 3, 2015

Xiaoping Du, MD, PhD

Pharmacology

835 S. Wolcott Avenue

403-E M.S.B., M/C 868

Chicago, IL 60612

Phone: (312) 355-0250 / Fax: (312) 996-1225

RE: Protocol # 1999-0610

"Mechanisms of Platelet Activation"

Dear Dr. Du:

Your Continuing Review was reviewed and approved by the Expedited review process on November 24, 2015. You may now continue your research.

Please note the following information about your approved research protocol:

Protocol Approval Period:	December 4, 2015 - December 3, 2016
Approved Subject Enrollment #:	1500 (1309 enrolled)

Performance Sites: UIC

Sponsor: a) NIH, b) NHLBI/NIH, c) NIH-National Institutes of Health, d) NIH/NHLBI, e) NHLBI - National Heart, Lung, and Blood Institute, f) National Heart, Lung and Blood Institute, g) National Institutes of Health

PAF#: a) 2009-05545, b) 2010-00091, c) 2011-02188, d) 2014-00591, e) 2013-00887, f) 2014-03153, g) 2014-04177

Grant/Contract No: a) 3R01HL068819-08S1, b) R01 HL062350, c) HL080264, d) F31HL123319, e) HHSN268201400007C, f) 2R01 HL062350, g) 1R01 HL125356-01

Grant/Contract Title: a) The cGMP-dependent protein kinase pathway in platelets, b) Signaling mechanism of platelet glycoprotein 1b-IX, c) Outside-in signaling mechanisms of platelet integrin alpha-llb-beta3, d) Investigation into the Mechanism of the thrombin receptor function of GPIb, e) Vascular Interventions/Innovations and Therapeutic Advances (VITA): Selective Inhibitors of Intergin Outside-in Signaling as a New Generation of Anti-Thrombotics, f) Signaling Mechanisms of Platelet GPIb-IX, g) New strategies for treating septic vasculopathy, inflammation and thrombosis

Research Protocol(s):

a) Mechanisms of Platelet Activation; Version 8; 11/05/2014

Recruitment Material(s):

a) Flyer, "Volunteers Needed...," Version 2, 03/12/2002
b) Protocol #1999-0610 - Advertisement for mass mail by Internet Version 2 05/06/2004
c) Flyer "Sickle cell patients needed to volunteer for research study", Version 3, 01/06/2011
d) Advertisement for mass mail by Internet for sickle cell research group, Version 1, 01/06/2011

Informed Consent(s):

a) "Mechanisms of Platelet Activation," Version 9, 11/16/2010
b) Consent/authorization for sickle cell patients, Version 1.0, 01/07/2011

HIPAA Authorization(s):

a) "Mechanisms of Platelet Activation," Authorization, Version 2, 04/01/2004

(Please continue to use the Authorization form approved and stamped 04/06/2004)

Additional Determinations for Research Involving Minors:

These determinations have not been made for this study since it has not been approved for enrollment of minors.

Your research meets the criteria for expedited review as defined in 45 CFR 46.110(b)(1) under the following specific category:

(2) Collection of blood samples by finger stick, heel stick, ear stick, or venipuncture as follows:

(a) from healthy, nonpregnant adults who weigh at least 110 pounds. For these subjects, the amounts drawn may not exceed 550 ml in an 8 week period and collection may not occur more frequently than 2 times per week; or

(b) from other adults and children, considering the age, weight, and health of the subjects, the collection procedure, the amount of blood to be collected, and the frequency with which it will be collected. For these subjects, the amount drawn may not exceed the lesser of 50 ml or 3 ml per kg in an 8 week period and collection may not occur more frequently than 2 times per week.

Please note the Review History of this submission:

Receipt Date	Submission Type	Review Process	Review Date	Review Action
11/20/2015	Continuing Review	Expedited	11/24/2015	Approved

Please remember to:

→ Use your **research protocol number** (1999-0610) on any documents or correspondence with the IRB concerning your research protocol.

→ Review and comply with all requirements on the guidance,

"UIC Investigator Responsibilities, Protection of Human Research Subjects"
(http://tigger.uic.edu/depts/ovcr/research/protocolreview/irb/policies/0924.pdf)

Please note that the UIC IRB has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

Please be aware that if the scope of work in the grant/project changes, the protocol must be amended and approved by the UIC IRB before the initiation of the change.

We wish you the best as you conduct your research. If you have any questions or need further help, please contact OPRS at (312) 996-1711 or me at (312) 413-0241. Please send any correspondence about this protocol to OPRS at 203 AOB, M/C 672.

Sincerely,

Ibraheem Oguntade

IRB Coordinator, IRB # 3

Office for the Protection of Research Subjects

Enclosure(s):

1. **Informed Consent Document(s):**
   a) "Mechanisms of Platelet Activation," Version 9, 11/16/2010
   b) Consent/authorization for sickle cell patients, Version 1.0, 01/07/2011

2. **Recruiting Material(s):**
   a) Flyer, "Volunteers Needed...," Version 2, 03/12/2002
   b) Protocol #1999-0610 - Advertisement for mass mail by Internet Version 2 05/06/2004
   c) Flyer "Sickle cell patients needed to volunteer for research study", Version 3, 01/06/2011
   d) Advertisement for mass mail by Internet for sickle cell research group, Version 1, 01/06/2011

cc: Asrar Malik, Pharmacology, M/C 868
OVCR Administration, M/C 672

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

April 29, 2016

Xiaoping Du
Pharmacology
M/C 868

Dear Dr. Du:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed and approved in accordance with the Animal Care Policies of the University of Illinois at Chicago on ***4/27/16.***

Title of Application: Signaling Mechanisms of Platelet Activation

ACC Number: 14-017

Modification Number: ***12***

Nature of Modification:

1) ***Addition of Personnel: Ying Liang***

2) ***Addition of 5/strain (Epsin1 fl/fl, Epsin1 fl/fl/Paf4 Cre , and Paf4 Cre) mice from Harvard for euthanasia and blood collection in quarantine and 15 WT controls (at UIC) to determine effect on platelet function.***

Condition of Approval: All work with mice must be in quarantine and addition modification required if further studies with new strains including breeding will be conducted.

Protocol Approved: ***3/28/2014***

Current Approval Period: ***2/18/2016*** to ***2/18/2017.***

Current Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 7

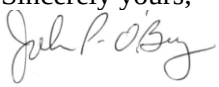
Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Signaling Mechanism of Platelet Glycoprotein Ib-Ix*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1 HL062350	*Funded*	*201000091*	*UIC*	*Xiaoping Du*

Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Outside-in signaling mechanisms of platelet integrin alpha-llb-beta3*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
HL-080264	*Funded*	*201102188*	*UIC*	*Xiaoping Du*
Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Selective Inhibitors of Integrin Outside- In Signaling as a New Generations of Anti-Thrombotics - Du, Xiaoping*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
HHSN268201400007C	*Funded*	*201300887*	*UIC*	*Xiaoping Du*
Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Investigation into the Mechanism of the Thrombin Receptor Function of GP1b*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
F31 HL123319	*Funded*	*201400591*	*UIC*	*Brian Estevez*
Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Signaling Mechanisms of Platelet GPIb-IX*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1 HL062350 (years 14-18)	*Funded*	*201403153*	*UIC*	*Xiaoping Du*
Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*New strategies for treating septic vasculopathy, inflammation and thrombosis*			*Portion of Grant is matched* *Only breeding- tied to ACC 14-153 for experiments*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
R01 HL 125356 (years 1-5)	*Funded*	*201404177*	*UIC*	*Xiaoping Du*
Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Outside-in Signaling Mechanisms of Platelet Integrin Alpha-IIb-Beta3*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
R01HL080264 (years 9-13) A1 version	*Pending*	*201601556*	*UIC*	*Xiaoping Du*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,



John P. O'Bryan, PhD
Chair, Animal Care Committee
JPO /*mbb*
cc: BRL, ACC File, Aleksandra Stojanovic

VITA

NAME

Zheng Xu

EDUCATION

2016, Master of Science, Pharmacology, University of Illinois, Chicago
2012, Bachelor of Science, Biotechnology, Beijing Normal University, China

HONORS AND AWARDS

2008, Undergraduate Scholarship, BNU

2010 Third Prize in Academic Papers Competition, BNU

2012 Beijing College Student Independent Research Fellowship, BNU

2016, CAAC Young Investigator Award (YIA), Chinese American Academy of Cardiology

2016, PVD Travel Award for Young Investigators, ATVB Scientific Sessions

PUBLICATION

Delaney, M. K., K. Kim, B. Estevez, **Z. Xu**, A. Stojanovic-Terpo, B. Shen, M. Ushio-Fukai, J. Cho, and X. Du. 2016. Differential Roles of the NADPH-Oxidase 1 and 2 in Platelet Activation and Thrombosis. *Arterioscler Thromb Vasc Biol* 36 (5):846-54.

Shen, B., B. Estevez, **Z. Xu**, B. Kreutz, A. Karginov, Y. Bai, F. Qian, U. Norifumi, D. Mosher, and X. Du. 2015. "The interaction of Galpha13 with integrin beta1 mediates cell migration by dynamic regulation of RhoA." *Mol Biol Cell* 26 (20):3658-70.

CONFERENCE PRESENTATIONS

Zheng Xu, M. Keegan Delaney, Kyungho Kim, Brian Estevez, Aleksandra Stojanovic-Terpo, Bo Shen, Masuko Ushio-Fukai, Jaehyung Cho, and Xiaoping Du. "Differential Roles of the NADPH-oxidase 1 and 2 in Platelet Activation and Thrombosis and a Common Syk/ PLC/Calcium-dependent ROS Signaling Pathway Mediating Platelet Activation." **ATVB|PVD 2016 Scientific Session**, Nashville, May 5-7, 2016

Zheng Xu, M. Keegan Delaney, Kyungho Kim, Brian Estevez, Aleksandra Stojanovic-Terpo, Bo Shen, Masuko Ushio-Fukai, Jaehyung Cho, and Xiaoping Du. "Differential Roles of the NADPH-oxidase 1 and 2 in Platelet Activation and Thrombosis and a Common Syk/ PLC/Calcium-dependent ROS Signaling Pathway Mediating Platelet Activation." **CAAC_ATVB Symposium**, Nashville, May 4, 2016