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NADPH OXIDASE 2 (NOX2): A KEY TARGET OF OXIDATIVE STRESS-MEDIATED PLATELET ACTIVATION AND THROMBOSIS

Running title: Clinical significance of platelet NOX2

By

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ABSTRACT

Oxidative stress represents an imbalance between the production of reactive oxygen species (ROS) and the cellular antioxidant system. Increased levels of oxidative stress contribute to the development of atherosclerosis that eventually leads to thrombosis; a principle cause of heart attacks and strokes. Thrombosis is a consequence of platelet activation and aggregate formation within the circulation. Platelet ROS are mostly generated by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NOX2 is an isoform from NADPH oxidase expressed in platelets and an important regulator of platelet activation-associated thrombosis. The present article aims to highlight the relative contribution of NOX2 as a key target of different platelet activation pathways and antiplatelet treatment.

Keywords: platelet; antiplatelet; oxidative stress; NADPH oxidase; NOX2.

1 INTRODUCTION

2 Cardiovascular diseases (CVD) continue to be a substantial health-care burden.

3 The recent decrease in cardiovascular mortality in high-income countries has been
4 associated with a rise in the numbers of aging patients living with CVD. Oxidative
5 stress is associated with several CVD, including hypertension, heart failure, stroke,
6 diabetes and atherosclerosis. The development of atherosclerosis in the arterial
7 circulation underlies the progress of thrombotic disease. Platelets are critical for
8 hemostasis, but under oxidative conditions, play also a key role in the process of
9 thrombosis (1).

10 Oxidative stress represents an imbalance between reactive oxygen species (ROS)
11 production and ROS removal by the cellular antioxidant defense system, whereby
12 they mediate damage to cell structures, including lipids, membranes, proteins and
13 DNA (2). Oxidative stress and ROS production have long been regarded as a key
14 pathophysiological mediator that ultimately leads to CVD. Chronic and acute
15 overproduction of ROS under pathophysiologic conditions is integral in the
16 development of CVD. In this context, ROS also participate in a wide variety of
17 pathophysiology processes such as elevated platelet activation (3).

18 Platelet activation is a complex process that involves different cellular signaling
19 pathways. Experimental and clinical studies support the pivotal role played by ROS
20 in elevated platelet activation. Collagen and thrombin-induced platelet aggregation
21 is associated with production of ROS, which acts as a second messenger by
22 stimulating the arachidonic acid metabolism and phospholipase C pathway (4-6). In

1 addition, ROS plays a key role in agonists (collagen, thrombin, or calcium
2 ionophore [A23187])-induced glycoprotein (GP) Iba ectodomain shedding (7).

3 Although ROS modulate signaling pathway during platelet adhesion and activation
4 does not individually activate GPIIb/IIIa receptors (8).

5 In platelets, ROS are mostly generated by reduced nicotinamide adenine
6 dinucleotide phosphate (NADPH) oxidase; an enzyme complex that was primarily
7 described in phagocytes. NADPH oxidase of the NOX family are important
8 enzymatic sources of ROS (9). Seven isoforms of NOX have been described in
9 mammals and the NOX2 isoform is expressed in platelets, where it induces
10 superoxide anion ($O_2^{\cdot-}$) production (10). The present article aims to highlight the
11 relative contribution of NOX2 as a key target of different platelet activation
12 pathways and antiplatelet treatment.

13 **OXIDATIVE STRESS AND PLATELET ACTIVATION**

14 Platelets have a dynamic functional repertoire with participation in hemostasis and
15 thrombosis, which is dependent on a complex balance of activatory and inhibitory
16 signaling pathways (11). Platelets play a key role in the prevention of excessive
17 blood loss through the formation of a thrombus. After vascular injury, platelets
18 rapidly adhere to activated endothelial cells and/or subendothelial matrix proteins
19 such as collagen and von Willebrand factor through receptor–ligand interactions.
20 Subsequently, activated platelets and the release of granular molecules such as
21 adenosine diphosphate (ADP), are essential for stable thrombus formation at the
22 site of vascular injury. However, excessive platelet activation and hyper-reactive

1 platelets are thought to contribute to atherothrombosis that can lead to myocardial
2 infarction and stroke (12, 13).

3 Platelets are equipped with an effective enzymatic antioxidant system, the most
4 important of which are superoxide dismutase (SOD), catalase (CAT), glutathione
5 peroxidase (GPX), glutathione transferase (GST) and glutathione reductase
6 (GSSG-R). However, the imbalance between the formation of ROS and
7 antioxidative system efficiency contributes to the pathogenesis of thrombotic
8 disease, through elevated intracellular levels of ROS, which mediate elevated
9 platelet activation (14).

10 In platelets, ROS including superoxide anion, hydroxyl radicals or hydrogen
11 peroxide act as second messengers to modulate platelets via calcium mobilization,
12 nitric oxide (NO) inactivation and through the interaction with arachidonic acid to
13 enable the formation of isoprostanes. Notably, elevated levels of isoprostanes are
14 associated with systemic and local platelet activation (15, 16).

15 During activation, platelets generate ROS through several intracellular sources
16 such as NADPH oxidase, cyclooxygenases, uncoupled endothelial nitric oxide
17 synthase (eNOS), xanthine oxidase (XO) and mitochondrial respiration (6, 17-19).
18 However, data concerning expression eNOS by platelets remain controversial.
19 Gambaryan *et al.*, demonstrate that human and mouse platelets do not
20 contain eNOS proteins or mRNA (20).

21 **NADPH OXIDASE AND PLATELET ACTIVATION**

1 Recently, it has become evident that NADPH oxidase activity is functionally
2 expressed not only in phagocytes but also in various cell types, including platelets.
3 Platelet NADPH oxidase is a multicomponent protein complex assembled by the
4 cellular subunits p47^{phox}, p67^{phox}, and the membrane-bound proteins p22^{phox} and
5 gp91^{phox}, which together with the small GTPase Rac1/2 associate to form the
6 active enzyme complex (21).

7 Platelet-associated NADPH oxidase mediates a thrombogenic phenotype. Growing
8 data from experimental and clinical studies provide evidence that NADPH oxidase
9 is implicated in altered platelet activation via superoxide anion production (22, 23).

10 Collagen activation induces NADPH oxidase-dependent superoxide anion release
11 in platelets, which in turn enhances the availability of released ADP, resulting in
12 increased thrombus formation (24). Indeed, NADPH oxidase inhibitors have been
13 shown to reduce platelet aggregation and thrombus formation on collagen under
14 high shear (25, 26). Meanwhile, inhibition with apocynin (NOX2 inhibitor that
15 prevents serine phosphorylation of p47^{phox} and blocks its association with gp91^{phox})
16 reduces platelet adhesion in patients with advanced atherosclerosis (27).

17 Seven distinct members of the NADPH oxidase family have been characterized, of
18 which four (namely NOX1, 2, 4 and 5) may have cardiovascular functions (28).

19 Among the enzymes generating ROS, NOX1 and NOX2 play differential roles in
20 different platelet activation pathways. Thus, using knockout mice, NOX1(-/Y)
21 platelets showed selective defects in thrombin- or thromboxane A₂ analog
22 U46619-mediated platelet activation.

NOX2 AND PLATELET ACTIVATION

NOX2 has been implicated in various aspects of CVD. It is suggested to play a role in favouring the occurrence of atrial fibrillation (AF) after cardiac surgery via formation of ROS, and in peripheral artery disease patients, ROS generated by NOX2 contribute to reduce flow-mediated dilation (29, 30). In addition, NOX2 promotes carotid plaque rupture and stroke occurrence (31). Meanwhile, administration of an antioxidant (propionyl-L-carnitine) is able to improve arterial dilatation via NOX2 inhibition (32).

Platelet-leukocyte interactions on activated endothelial cells play an important role during microvascular occlusion under oxidative stress conditions. Platelet NOX2-produced ROS regulated P-selectin exposure upon agonist stimulation and the ligand-binding function of GPIIb/IIIa, which contribute to the pathology of hepatic ischemia/reperfusion injury during vascular inflammation (33). Furthermore the activation of NOX2 on platelets is increased in heart failure, likely as a consequence of the underlying inflammatory processes (34).

NOX2 is expressed in platelets and the megakaryocyte cell-line (MEG01). In human platelets the enzymatic activity of NOX2 believed to be an important source of ROS in platelets (10, 21, 35). NOX2-generated superoxide anion is rapidly converted into the longer-lasting and membrane diffusible hydrogen peroxide, which is the major ROS contributing to pathological signaling through oxidative modification of lipids and proteins (24, 36). This is evidenced by the almost absent ROS production by platelets from patients with hereditary deficiency of NOX2 (37,

38). The platelet NOX2 enzyme consists of membrane subunits (p22^{phox} and gp91^{phox}) and cytosolic components (p47^{phox}, p67^{phox} and p40^{phox}), which together with the small GTPase rac1/2 associate to form the active enzyme complex (Figure 1) (21, 36, 39). Upon activation, binding of Rac1 GTPase to p67^{phox} plays a critical role in NOX2 activation by facilitating the assembly of the NOX2 enzyme complex. Meanwhile, inhibition of Rac1 GTPase by NSC23766 or gene targeting on Rac1 GTPase clearly demonstrated that Rac1 is essential for agonist induced ROS generation in platelets (40, 41). Therefore, NOX2 could be a key target of different platelet activation pathways. The pathways modulated by platelet NOX2 are illustrated in Figure 1.

a) Activation pathways of platelet NOX2

NOX2 can be activated by sCD40L/CD40L, collagen/GPVI or ox-LDL/CD36.

sCD40L/CD40L. CD40 ligand (CD40L) is expressed and functional on platelets, and plays a pivotal role in atherosclerosis (42). CD40L is cryptic in unstimulated platelets but is rapidly unveiled on the platelet surface after platelet stimulation (43). Platelet production of superoxide anion plays a key role in CD40L expression and platelets from patients with an inherited deficiency of gp91^{phox} exhibited defects in superoxide anion production and CD40L expression (36). In addition, such patients exhibit very low levels of sCD40L in unstimulated platelets and no changes following platelet stimulation (36). This suggests that the surface-expressed CD40L is subsequently cleaved over a period of minutes to hours, generating a soluble fragment termed sCD40L that remains trimeric. Indeed, it has

1 been calculated that >95% of circulating sCD40L originates from platelets (43).
 2 Elevated levels of sCD40L enhance platelet activation, aggregation, and platelet-
 3 leukocyte conjugation, and increase stimulation-induced platelet increase of ROS
 4 through activation of Akt and p38 MAP kinase signaling pathways (44). In this
 5 context, NOX2 is an important mediator in sCD40L-induced ROS generation.
 6 Consistent with this, apocynin inhibits the increase of ROS induced by sCD40L
 7 (44). Moreover, plasma levels of sCD40L are dependent on the expression of
 8 NOX2. Additionally sCD40L is reduced in gp91^{phox} defective patients and is
 9 expressed at higher levels in obese women where NOX2 is upregulated (45).

10 **Collagen/GPVI.** GPVI is a membrane glycoprotein unique to platelets and has
 11 been identified as a physiological receptor for collagen (46). NOX2 subunit p47^{phox}
 12 and binding partner tumor necrosis factor receptor associated factor (TRAF) 4 are
 13 associated with the cytoplasmic tails of GPVI, and link ROS production to NOX2
 14 function (47). TRAF4 is immediately upstream of a proline-rich sequence that
 15 binds the tyrosine kinase Lyn. Lyn is involved in phosphorylation of the
 16 immunoreceptor tyrosine-based activation motif (ITAM) motif within the Fc receptor
 17 (FcR) γ -chain (in complex with GPVI), leading to activation of Syk and the Syk-
 18 dependent signaling pathway involving SLP76, Bruton's tyrosine kinase (BTK),
 19 phosphatidylinositol 3-kinase (PI3K), phospholipase C (PLC) and protein kinase C
 20 (PKC). GPVI stimulation culminates in affinity up-regulation of GPIIb/IIIa allowing
 21 fibrinogen binding and thus, platelet aggregation and thrombus formation (47).
 22 NOX2 (-/-) platelets showed reduced ROS generation and potent inhibition of CRP-

1 induced platelet activation. Also, a partial inhibition of thrombin-
2 induced platelet activation was observed (22).

3 **ox-LDL/CD36.** Oxidized lipids are markers of oxidative stress, important mediators
4 of atherosclerosis and activators of platelets. Metabolic syndrome in obese
5 patients, diabetic or non-diabetic, is associated with increased oxidative stress in
6 low-density lipoprotein (ox-LDL), which triggers platelet activation (48, 49). In
7 addition, a low HDL phenotype, both in CVD patients and healthy subjects, is
8 associated with increase of lipid peroxidation and platelet activation (50).

9 A number of studies have suggested that ox-LDL may bind to and activate platelets
10 (51-53). Platelet CD36, a member of the Type 2 scavenger receptor family, is a
11 multiligand pattern recognition receptor that recognizes specific oxidized
12 phospholipids, molecules expressed on microbial pathogens, apoptotic cells, and
13 cell-derived microparticles. Elevated platelet CD36 expression may contribute to
14 increased risk of thrombo-embolism. The interactions of platelet CD36 with
15 endogenous oxidized lipids play a crucial role in the prothrombotic phenotype (54).
16 CD36 binds ox-LDL and induces platelet hyperactivity via generation of ROS. The
17 synthesis of ROS by ox-LDL/CD36 required Src-family kinases, PKC-dependent
18 phosphorylation and activation of NOX2, which is blocked by CD36 inhibitors,
19 NOX2 inhibitor (gp91ds-tat) and is absent in NOX2(-/-) mice (51). Therefore
20 platelet activation via specific ox-LDL/CD36 is mediated by NOX2 activation (55).
21 Moreover, at pathophysiological levels in hyperlipidemic patients, oxidized choline

glycerophospholipids are able to bind and promoted platelet activation via CD36 (54).

b) Downstream signaling pathway of platelet NOX2 activation

Downstream of NOX2, ROS production occurs, thromboxane A₂ levels are increased and P-selectin expression and intracellular Ca²⁺ release occur. Intracellular Ca²⁺ release modulates an early stage of cell activation such as granular secretion. In addition, platelet NOX2-derived ROS, have been shown to mediate the oxidation of sulfhydryl groups in GPIIb α and enhance its ligand-binding function with von Willebrand factor on endothelial cells and the integrin α M β 2 on monocytes (33, 56).

The 8-iso-prostaglandin F₂ α (8-iso-PGF₂ α) is a stable isoprostane and reliable marker of oxidative stress *in vivo* (57). Elevated levels of 8-iso-PGF₂ α in CVD are associated with systemic and local platelet activation and notably, platelet NOX2 contributes to the formation of 8-iso-PGF₂ α (45, 58). In children with hypercholesterolemia, platelet gp91^{phox} and urinary isoprostanes levels are increased (38). Platelet 8-iso-PGF₂ α formed as a consequence of NOX2 activation contributes to platelet recruitment via activation of GPIIb/IIIa (39).

CLINICAL SIGNIFICANCE OF PLATELET NADPH

Excessive ROS production by NADPH oxidase causes cellular stress, leading to various diseases, including thrombotic conditions. Therefore, a complete understanding of the function of platelet-derived NADPH oxidase is important to direct the role of this enzyme towards antiplatelet therapy.

1 Patients with the clinical syndrome of chronic granulomatous disease (CGD) have
2 increased propensity to infection with certain bacteria. Importantly, investigation of
3 X-linked CGD patients have provided a clinical model to study the role of NADPH
4 in the atherothrombosis and its clinical sequelae. In this context, CGD patients with
5 gp91^{phox} or p47^{phox} deficiency have an impaired inactivation of NO would result in
6 enhanced NO bioavailability and arterial dilatation. In addition, these patients had
7 significant reduction of carotid intima–media thickness with a similar reduction
8 detected in both p47^{phox}- and gp91^{phox}-deficient subtypes. In contrast, the
9 prevalence of coronary arterial calcification was similar between patients with CGD
10 and controls. These finding suggests that loss of NADPH function prevents or
11 retards atherosclerosis progression even in a disease such as CGD (59).

12 Platelets from NOX2(-/-) patients displayed defective arterial thrombosis but
13 bleeding time was not affected in CGD patients, suggesting an important role for
14 platelet NOX2 in thrombosis but not in hemostasis (22, 55). Thus platelet
15 aggregation of platelets obtained from CGD patients was significantly reduced in
16 association with impairment of 8-iso-PGF2 α production. In addition, NOX2(-/-)
17 platelets showed potent inhibition of collagen-related peptide (CRP, a GPVI
18 selective agonist)-induced platelet activation, and also showed partial inhibition of
19 thrombin-induced platelet activation. Consistent with these findings, platelets from
20 gp91^{phox}-deficient (catalytic core of NOX2) patients showed an almost complete
21 absence of superoxide anion and CD40L expression, but collagen-induced platelet
22 aggregation was similar to that of healthy subjects (36).

1 ROS formed by NOX activity play a critical role in CVD, indeed, the C242T
2 polymorphism of the NOX gene is a pathogenetic risk factor for CVD (60).
3 Tamoxifen is effective in the prevention and treatment of breast cancer, but its use
4 is associated with an increased risk of thrombosis. Incubation of platelets with the
5 active metabolites of tamoxifen increases stimulation-dependent superoxide
6 anion release through a NADPH oxidase-dependent mechanism and therefore
7 may cause thrombosis (61).

8 Up to one-third of serious vascular events in high-risk patients are attributable to
9 aspirin resistance. In platelets from aspirin-resistant patients, both NADPH-driven
10 superoxide anion production, and expression of gp91^{phox} and p67^{phox} subunits
11 tended to increase. Maximal aggregation of aspirin resistant platelets to collagen
12 and epinephrine was significantly decreased by NADPH oxidase inhibitors
13 (diphenylene iodonium and apocynin), whereas they had no effect in aspirin
14 sensitive platelets. These data suggest a potential use of NADPH oxidase
15 inhibitors in the occurrence of thrombosis in high-risk cardiac patients (56, 62).

16 Since NOX2 is expressed in tissues throughout the cardiovascular system, and
17 plays an important role in platelet activation in thrombosis but not hemostasis,
18 platelet NOX2 inhibition might represent a promising strategy to prevent
19 thrombosis in both aspirin resistant and tamoxifen treated patients (22, 34, 63). As
20 shown in Table 1, platelet NOX2 inhibitors could be used in the prevention of CVD
21 via inhibition of platelet activation. The main mechanisms inhibitors of NOX2 are
22 compounds which bind to p67^{phox}, p47^{phox} or Rac1; thereby preventing assembly of

the active NOX2 complex (64-69). These findings may provide a rationale for the use of perhexiline and statins to prevent platelet activation via inhibition of NOX2.

CONCLUSION

There is abundant evidence that oxidative stress, caused by an excess of ROS, regulates several components of thrombosis, including platelet activation. NOX2 is an isoform from NADPH oxidase expressed in platelets and is likely one of the important sources of oxidative stress. Platelet NOX2 is a key target of different platelet activation pathways, and may represent an alternative or additional therapeutic target to prevent thrombosis associated with oxidative stress.

Practice Points:

NOX2 plays a prominent role in platelet activation.

NOX2 can be activated by sCD40L/CD40L, collagen/GPVI or ox-LDL/CD36.

Perhexiline (an approved prescription drug for angina) and statins (atorvastatin and rosuvastatin) used clinically present inhibition of NOX2 and antiplatelet activity

Research Agenda:

Clinical value of platelet NOX2 inhibitors.

Experimental studies on side effects of platelet NOX2 inhibitors.

Conflict of interest

The authors have no conflicts of interest to disclose.

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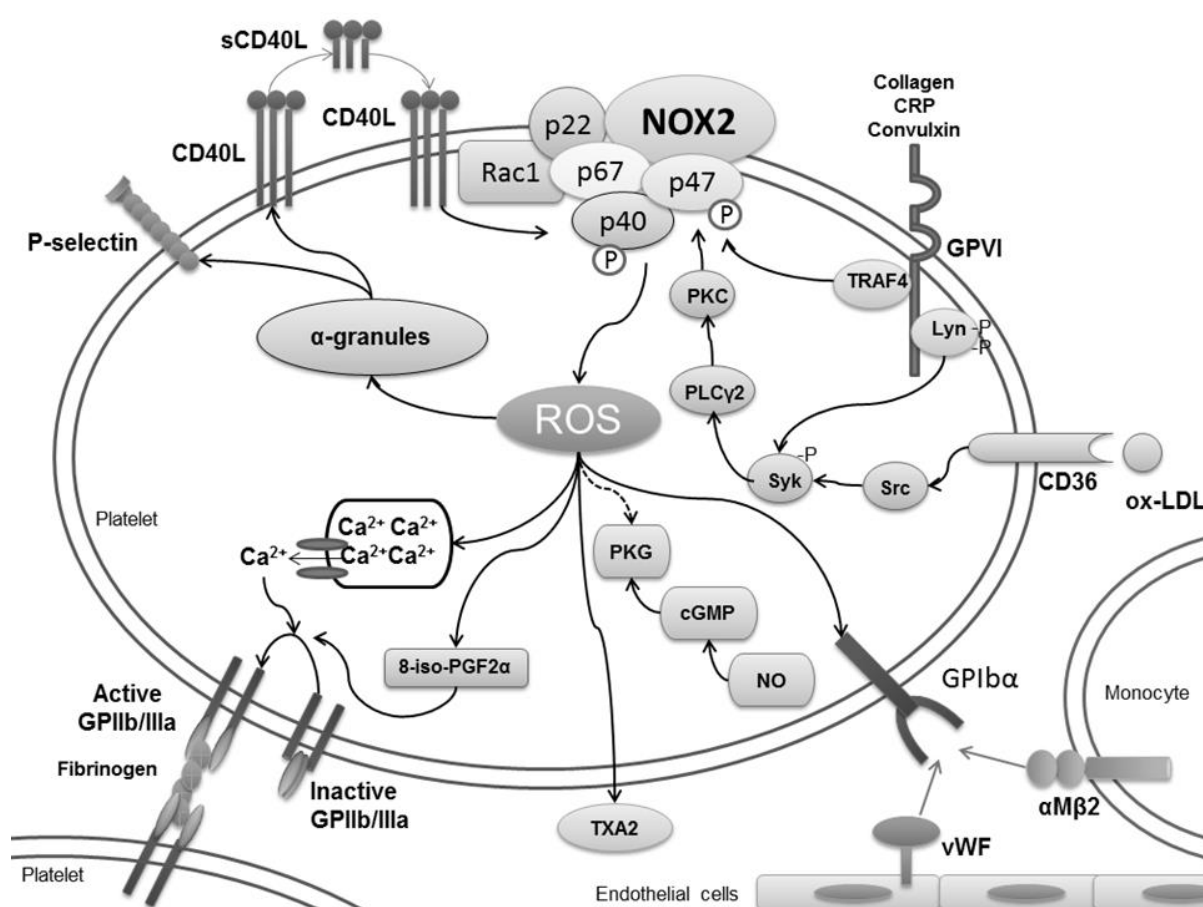
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1 **Table 1.** Inhibitors of platelet NOX2 with antiplatelet activity.

Drugs	NOX2 inhibition	Antiplatelet activity
Phox-I1	Phox-I1 binds to p67 ^{phox} and abrogates Rac1 binding.	Platelets treated with Phox-I inhibited CRP, thrombin or U46619 induced ROS generation, P-selectin expression, ATP secretion and aggregation.
Celastrol	Celastrol bound to SH3 domain of p47 ^{phox} and disrupted the binding of the proline rich region of p22 ^{phox} .	Celastrol inhibited ADP or thrombin-induced expression of P-selectin and GPIIb/IIIa, and ADP-stimulated platelet fibrinogen binding.
Ebselen	Ebselen interrupted the translocation of p47 ^{phox} to membranes.	In aspirin-treated human platelets ebselen inhibited agonist-triggered increase in intracellular calcium.
NOX2ds-tat	NOX2ds-tat binds to p47 ^{phox} , thereby preventing assembly of the active NOX2 complex.	NOX2ds-tat inhibited platelet oxidative stress and function including platelet ROS, NOX2 activation, 8-iso-PGF2 α formation and platelet recruitment.
Suramin	Inhibited biochemical NOX2 activity.	Suramin inhibited platelet aggregation induced by thrombin, PAF, ALPA, or arachidonic acid and increase in intracellular calcium.
Perhexiline	Inhibited biochemical NOX2 activity.	Perhexiline increase NO/cGMP in SAP and ACS patients.
Statins (atorvastatin and rosuvastatin)	Reduce the expression of Rac1, p22 ^{phox} and gp91 ^{phox} .	Inhibited platelet activation and isoprostanes, TXA2 and platelet O ₂ ⁻ release.

- 2 ACS: acute coronary syndromes, ADP: adenosine diphosphate, ALPA: alkyllysophosphatidic acid, ATP: adenosine
- 3 triphosphate, CRP: collagen-related peptide, cGMP: cyclic guanosine monophosphate, NO: nitric oxide, PAF: platelet
- 4 activating factor, ROS: reactive oxygen species, SAP: stable angina pectoris, TXA2: thromboxane A2, 8-iso-PGF2 α : 8-iso-
- 5 prostaglandin F2 alpha.
- 6



1

2 **Figure 1.** Activation and downstream signaling pathways of platelet NOX2.

3 Continuous lines: activation and dotted line: inhibition.