Platelet NOX, a novel target for anti-thrombotic treatment

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Summary
There is a growing body of evidence to suggest that reactive oxidant species (ROS) including O$_2^-$, OH$^-$ or H$_2$O$_2$ act as second messengers to activate platelets via 1) calcium mobilisation, 2) nitric oxide (NO) inactivation, and 3) interaction with arachidonic to give formation of isoprostanes. Among the enzymes generating ROS formation NOX2, the catalytic core of NADPH oxidase (NOX), plays a prominent role as shown by the almost absent ROS production by platelets taken from patients with hereditary deficiency of NOX2. Experimental and clinical studies provided evidence that NOX2 is implicated in platelet activation. Thus, impaired platelet activation has been detected in patients with NOX2 hereditary deficiency. Similarly, normal platelets added with NOX2 specific inhibitors disclosed impaired platelet activation along with ROS down-regulation. Accordingly, animals prone to atherosclerosis treated with apocynin, a NOX inhibitor, showed reduced platelet adhesion and atherosclerotic plaque. Furthermore, a significant association between NOX2 up-regulation and platelet activation has been detected in patients at athero-thrombotic risk, but a cause-effect relationship needs to be established. These findings may represent a rationale to plan interventional trials with NOX inhibitors to establish if blocking NOX2 or other NOX isoforms may represent a novel anti-platelet approach.

Keywords
Platelets, oxidative stress, NOX, reactive oxygen species

Introduction
Reactive oxidant species (ROS) are chemically unstable molecules, which rapidly react with other molecules inducing formation of oxidised products such as oxidised low-density lipoprotein (LDL), peroxynitrite or protein adducts. At physiologic concentration ROS serve as second messengers and, as such, they behave as intracellular signals for cell activation. Among the cells in which ROS act as second messengers, platelets represent a typical example of ROS involvement in the activation process. Thus, upon stimulation by common agonists, platelets produce several types of ROS such as superoxide anion (O$_2^-$) or hydrogen peroxide (H$_2$O$_2$), that in turn contribute to propagation of platelet aggregation (2). The knowledge of mechanisms which up-regulate, and the knowledge of therapeutic approach which modulates ROS formation may have relevance in the setting of athero-thrombosis. There are several enzymatic pathways which elicit formation of ROS into the cells including NADPH oxidase (NOX), myeloperoxidase, xanthine oxidase or uncoupled nitric oxide (NO) synthase. Amid these, experimental and clinical evidence focus on the role of NOX as key enzyme in producing ROS, activating platelets via 1) calcium mobilisation, 2) nitric oxide (NO) inactivation, and 3) the clinical settings in which inhibition of NOX may be of potential interest to prevent atherothrombotic complications.

NOX: from innate immune-system to athero-thrombosis
NOX is an enzymatic system that transfers electrons across biological membranes. In general, the electron acceptor is oxygen and the product of the electron transfer reaction is O$_2^-$; therefore, the biological function of NOX is the generation of ROS (6). The main common structural features include 1) a NOX-binding site, 2) a FAD-binding region, 3) six conserved trans-membrane domains, and 4) four highly conserved haeme-binding histidines. NOX has been primarily described in white cells where it greatly contributes to the immune system activity via production of ROS resulting in bacteria killing (7, 8). Phagocytic NOX comprises a catalytic subunit, gp91phox (also known as NOX2) that, in conjunction with a gp22phox subunit, forms a membrane-bound heterodimeric flavocytochrome b558. NOX2 is unstable in the absence of p22phox; phagocytes from p22phox-deficient patients have no detectable NOX2 protein activity (9-11). Activation of NOX2 requires translocation of cytosolic factors, namely p47phox, p67phox, p40phox and...
RAC to the NOX2/p22\textsubscript{phox} complex. Phosphorylated p47\textsubscript{phox} interacts with p22\textsubscript{phox} (12, 13). p47\textsubscript{phox} organises the translocation of the other cytosolic factors, hence it is designated as the "organiser sub-unit." p47\textsubscript{phox} brings the "activator subunit" p67\textsubscript{phox} into contact with NOX2 along with the small subunit p40\textsubscript{phox} (14). Finally, the GTPase Rac interacts with NOX2 via a two-step mechanism involving an initial direct interaction with NOX2 (15), followed by a subsequent interaction with p67\textsubscript{phox} (16). NOX2 is also present in endothelial cells (17), cardiomyocytes (18), haematopoietic stem cells (19) and platelets (20). In case of genetic deficiency of NOX2-derived ROS patients experience a serious disease (chronic granulomatous disease, CGD), which is characterised by life-threatening infections; this trend to infections is due to impaired killing bacteria property by white cells, which is related to lower or absent ROS production (7, 8).

Several NOX isoforms have been reported to exist in cells other than phagocytes in the cardiovascular system, with significant differences in the organisation and mechanisms of activation. NOX1 shares a 60% high degree of sequence identity with NOX2 (21). NOX1 was identified in vascular smooth muscle cells (22, 23) and endothelial cells (24). O\textsubscript{2}\textsuperscript{-} generation by NOX1 requires the membrane subunit p22\textsubscript{phox} and cytosolic subunits similarly to NOX2 (25, 26). The NOX1 specific cytosolic sub-units were named NOXO1 (NOX organiser 1: analogue to p47\textsubscript{phox}) and NOXA1 (NOX activator 1: analogue to p67\textsubscript{phox}). Nevertheless NOX1 is also able to use the p47\textsubscript{phox} and p67\textsubscript{phox} subunits (21). There is also ample evidence for the involvement of the small GTPase Rac in the regulation of NOX1 activity (27).

NOX4 was identified in endothelial cells (28), smooth muscle cells (29) and fibroblasts (30). NOX4 is a p22\textsubscript{phox} -dependent enzyme and functional studies demonstrate a p22\textsubscript{phox} requirement for NOX4-dependent ROS generation (26). Differently from NOX2, active NOX4 does not require either cytosolic subunits or cell stimulation (31-33). The peculiarity of NOX4 is in the formation of hydrogen peroxide, rather than superoxide, by a specific third extra-cytosolic loop that is not present in NOX1 and NOX2 (33, 34).

The last described NOX in the cardiovascular system is NOX5 that is expressed in vascular smooth muscle (35). Activation of NOX5 does not require either p22\textsubscript{phox} (26) or cytosolic organiser and activator subunits (36) and works even in cell-free systems (37). NOX5 activation is mediated by a rise in the cytoplasmic calcium concentration that is bound by a specific calcium-binding domain on NOX5 (37).

Regarding platelets, studies so far reported showed that platelets express only NOX2 (Figure 1). Thus, Seno et al. first showed that platelets possess sub-units of NOX by demonstrating the presence of the membrane p22\textsubscript{phox} and the cytosolic p47\textsubscript{phox} in platelet lysates (38). Afterwards we demonstrated that platelets express the catalytic sub-unit gp91\textsubscript{phox} on platelet surface so providing further support to the role of platelets on the production of ROS and eventually on innate immune system (4); other components of NOX2 encompassed by platelets included p47\textsubscript{phox}, p67\textsubscript{phox} and Rac (4, 20, 38). Activation of platelet NOX is crucial for O\textsubscript{2}\textsuperscript{-} production as shown by its almost complete suppression in case of NOX2 hereditary deficiency. This has been evidenced in patients with X-CGD, whose platelets disclosed severe but not complete suppression of platelet O\textsubscript{2}\textsuperscript{-} as a consequence of the hereditary deficiency of NOX2 (20). Although incomplete suppression of platelet O\textsubscript{2}\textsuperscript{-} suggest that other NOX isoforms may contribute to such residual platelet ROS production, a recent study failed to show either NOX1 or NOX4 in platelets (37). This does not exclude, however, that other NOX isoforms may be detectable on platelet surface, therefore further study needs to be done to explore this issue.

**Figure 1: NOX structure and conformational changes upon activation.** NOX activation requires the formation of a specific complex between the membrane subunits and the cytoplasmic subunits.

**NOX-derived ROS and platelet aggregation**

NOX-derived ROS have an important impact on platelet function. Krotz et al (39, 40) demonstrated that NOX-dependent platelet O\textsubscript{2}\textsuperscript{-} formation enhances platelet aggregation and platelet-dependent thrombosis. In accordance with this finding we observed that in subjects with genetically determined impaired O\textsubscript{2}\textsuperscript{-} formation, agonist-induced platelet aggregation was reduced, further supporting the role of O\textsubscript{2}\textsuperscript{-} in eliciting platelet activation (4). Once generated O\textsubscript{2}\textsuperscript{-} is rapidly converted by superoxide-dismutase (SOD) to H\textsubscript{2}O\textsubscript{2}, which is chemically stable. Thus, while O\textsubscript{2}\textsuperscript{-} is charged, hardly permeable and short-lived, H\textsubscript{2}O\textsubscript{2} is uncharged and diffusible and has a longer half-life (41). Platelet production of O\textsubscript{2}\textsuperscript{-} may act as second messenger to elicit H\textsubscript{2}O\textsubscript{2} and F\textsubscript{2}-isoprostanes (see below) generation or by interfering with NO activity/biosynthesis (4) (Figure 2).

As far as second messenger activity is concerned, NOX-derived O\textsubscript{2}\textsuperscript{-} may affect platelet activation via PLA\textsubscript{2}-dependent arachidonic acid release from platelet membrane. This has been demonstrated by the ability of the specific PLA\textsubscript{2} inhibitor A23187 to inhibit anoxia-induced platelet activation, which is known to be mediated by platelet ROS formation (42). As second messenger, O\textsubscript{2}\textsuperscript{-} could also act through a SOD-dependent dismutation into H\textsubscript{2}O\textsubscript{2} (43). Thus, collagen-induced platelet aggregation is associated with a burst of H\textsubscript{2}O\textsubscript{2}, which in turn serves to activate platelets via intracellular calcium mobilisation (43). Downstream effects of H\textsubscript{2}O\textsubscript{2}-induced calcium mobilisation include production of arachi-
donic acid release from platelet membrane, thromboxane A₂ and PLC up-regulation (43). The crucial role for H₂O₂ in eliciting platelet activation has been recently confirmed and extended by Dayal et al. in an in vivo model of thrombosis performed in wild-type C57BL/6J mice or mice over-expressing glutathione peroxidase-1 (Gpx1). This is an enzyme which detoxifies the cells from H₂O₂ (3). All the experiments were performed in aged animals (3). Thus, using two models of artery and venous thrombosis, they showed a longer time to artery occlusion and a lower susceptibility to venous thrombosis in mice over-expressing Gpx1 compared to wild type. Platelets from wild-type aged mice showed platelet over-activation, which was associated with enhanced platelet H₂O₂ formation and was inhibited by catalase, an enzyme that destroys H₂O₂ (44). Platelet aggregation inhibition by catalase was associated with down-regulation of glycoprotein (Gp) IIb/IIIa suggesting that Gp Ib/IIa activation may be the mechanism through which H₂O₂ activates platelets. To explore if H₂O₂-mediated platelet aggregation was dependent upon NOX activation, platelets from wild-type mice were treated with apocynin, which determined lower GpIIb/IIa activation indicating that H₂O₂-mediated GpIIb/IIa activation was dependent upon NOX regulation. Of note, platelets with H₂O₂ over-production expressed significant levels of mRNA for the catalytic sub-unit Nox2 and the cytosolic sub-unit p47phox but not for Nox1 and Nox4 (3).

These findings are consistent with previous data from another experiment model that tested, in vivo, the role of H₂O₂ in platelet-dependent thrombosis. The study has been conducted in animals deficient of Gpx3, which is the only isoform of the Gpx family exclusively found in the extracellular compartment (45). Gpx3 is able to scavenge ROS likely including H₂O₂ (46). Animals deficient of Gpx3 displayed shorter bleeding time and enhanced platelet aggregation response to ADP compared to wild-type and more susceptibility to in vivo platelet activation and thrombosis. Also, animals deficient of Gpx3 were farther prone to cerebral infarction and platelet-dependent strokes in a no-flow ischaemia/reperfusion stroke model (45). These findings are in keeping with a study reporting Gpx3 deficiency in two brothers who suffered from arterial thrombosis and stroke (3, 47). Both patients had enhanced platelet activation in vitro with lowered response to NO and about three-fold increase of plasma H₂O₂ levels (3, 47).

O$_2^-$ may also modulate platelet function by interfering with extracellular mechanism implicated in platelet activation. Thus O$_2^-$ mainly interacts with NO so inactivating it and giving formation of peroxide nitrite; in this context NOX-derived O$_2^-$ serves to modulate platelet function by favouring platelet formation and thrombus growth. Thus, NO is an important molecule produced by endothelial cell responsible for the inhibition of platelet aggregation (48). NO is synthesised by the family of NO synthases which exist as endothelial, neuronal and inducible forms and acts as a paracrine signalling molecule in cardiovascular system (49). NO can act on platelets by diffusing across the plasma membrane of cells (50). NO-sensitive guanylyl cyclase (NO-GC) is the principal receptor for NO in platelets as recently demonstrated in a knock-out mice model for NO-GC where the inhibitory effect of NO on platelet function was completely abolished (51). In this experimental model administration of several NO donors, even at millimolar concentration, was unable to affect platelet activation (51).

NO synthase has been found also in platelets and its interplay with platelet NOX has been recently underscored in patients with CGD, who are characterised by hereditary deficiency of NOX2 (50) (4). Thus, this clinical setting is associated with severe suppression of platelet O$_2^-$ and increased platelet levels of NO (4).

**NOX, F2-iso-P and platelet activation**

Platelet ROS formation are also relevant in the process of platelet activation with a mechanism including non-enzymatic oxidation...
of arachidonic acid to give formation of platelet F2-isoprostanes (52). Formation of F2-isoprostanes complements that of thromboxane (Tx) A2, which is an unstable aggregating eicosanoid formed via cyclooxygenase (COX)-1 activation implicated in the thrombotic process (53). Conversely, F2-isoprostanes in particular 8-iso-PGF2α, is a chemically stable compound derived from non-enzymatic oxidation of arachidonic acid (54). The key role played by ROS, in particular O2·−, in the formation of F2-isoprostanes has been supported by impaired platelet 8-iso-PGF2α formation in case of hereditary deficiency of NOX2 (4).

Previous studies have shown that upon stimulation platelets release 8-iso-PGF2α in a concentration depending on the type of agonist used, but usually in the range of 100–500 pmol/l (55–57). We found that aspirin inhibited 8-iso-PGF2α formation by 8%, which is consistent with previous data showing a marginal role for COX-1 in the formation of this isoprostane (4). Conversely a much higher inhibition (~58%) was observed with a specific inhibitor of NOX2, suggesting that NOX has a major role in 8-iso-PGF2α formation (4).

Furthermore 8-iso-PGF2α was significantly reduced in patients with NOX2 hereditary deficiency compared to controls with a mean reduction of 75%, indicating that platelet generation of 8-iso-PGF2α is maximally dependent upon non-enzymatic oxidation of arachidonic acid via NOX2-derived O2·− (4).

While the role of TxA2 in the activation of platelets is well established, data concerning the role of 8-iso-PGF2α is less clear. In order to explore the role of F2-isoprostane on platelet activation we studied the effect of 8-iso-PGF2α in the process of platelet recruitment, that mimics in vivo accumulation of platelets at site of vascular injury (58). Recruitment is influenced by aggregating molecules that propagate thrombus plug upon release by platelets that originally initiated the aggregation (58). In platelets from patients with X-CGD 8-iso-PGF2α production was reduced and associated with impaired platelet recruitment. Also, incubation of platelets from X-CGD patients with 8-iso-PGF2α resulted in a partial restoration of platelet recruitment (4). Finally, we analysed the role of NOX2-dependent 8-iso-PGF2α formation on platelet activation in an in vitro model of shear-induced thrombus formation and found a reduced platelet thrombus formation upon blood perfusion at 1,500 s−1 in samples where isoprostane formation was suppressed by the NOX inhibitors apocynin and gp91ds-tat (4).

Pharmacologic studies

The pharmacologic armamentarium which inhibits NOX and in turn platelet aggregation is still scarce; it includes non-specific inhibitors of NOX such as statins and polyphenols and specific inhibitors such as apocynin (see Figure 2).

As far as statins are concerned, in vitro and in vivo pharmacologic studies with atorvastatin and rosuvastatin demonstrated that they are capable of inhibiting platelet NOX2 and in turn platelet aggregation. Thus, we measured several markers of platelet activation as early as 2 hours (h) from 40 mg of atorvastatin in hypercholesterolemic patients and showed (5) an immediate down-regulation of NOX2 and isoprostanes along with inhibition of platelet aggregation. This led to hypothesise that statins exert an anti-platelet effect with an oxidative stress-mediated mechanism via NOX2 down-regulation. In vitro study confirmed this hypothesis as atorvastatin dose-dependently (0.1–10 μM) down-regulated NOX2-derived oxidative stress, ultimately leading to impaired platelet activation and isoprostane formation (5). Similar results were achieved in vitro and in vivo with rosuvastatin (5, 59). Administration of either drug was associated not only with impaired NOX2-derived oxidative stress but also with enhanced platelet generation of NO, which is consistent with a similar effect elicited by statins in other cells such as endothelial cells (60).

Polyphenols have been also shown to affect platelet NOX. In vitro incubation of platelets with scalar concentrations of two polyphenols, namely catechin and quercetin, synergistically inhibited platelet NOX2 and ROS, an effect that was associated with impaired platelet activation so suggesting that polyphenols inhibited platelet activation via NOX2-downregulation (61, 62). This effect was further corroborated by in vivo study consisting in measuring platelet activation in subjects given 40 g dark or milk chocolate (63). The study showed that 2 h after administration of dark chocolate, which is, differently from milk chocolate, rich in polyphenols (64), platelet activation was significantly inhibited coincidentally with impaired NOX2-derived ROS (63).

Apocynin is a molecule that interferes with NADPH oxidase activation by inhibiting p47phox translocation to the catalytic subunit (65). In vitro studies with different methods to measure platelet activation consistently showed that apocynin inhibits platelet activation (66, 67). In particular, apocynin impaired ROS formation by agonist-stimulated platelets and significantly reduced platelet recruitment (68). This effect was associated with lower production of platelet isoprostanes indicating that inhibition of NOX2 activation ultimately results in down-regulating the expression of these platelet eicosanoids (68). Consistent with these findings, experimental study in animals demonstrated that apocynin impairs platelet function also in vivo. Thus, mice prone to atherosclerosis were studied at 30 weeks and again after 10 weeks with or without apocynin (3). Molecular imaging in non-treated animals demonstrated enhanced platelet activation as shown by a two-fold increase in P-selectin and platelet adhesion (3). Such changes were lowered in animals treated by apocynin in a dose-dependent fashion lending to suggest that inhibition of NADPH oxidation is associated with reduction of platelet activation.

Clinical studies

There are very few studies that analysed the interplay between NOX and platelet activation in vivo. Clinical settings include patients who underwent percutaneous coronary intervention (PCI), which is complicated by an ischaemia/reperfusion process and eventually by a burst of ROS (69) and patients at risk of atherosclerosis, including type II diabetes mellitus (70). These are all associated with cellular and systemic signs of oxidative stress (66, 71).

Experiments conducted before and after PCI demonstrated that, despite aspirin treatment, patients showed an increase platelet
production of TxB₂, which may reflect over-activation of platelet COX1 (69). Such increase occurred simultaneously to serum NOX2 elevation suggesting a potential interplay between NOX2 expression and TxB₂ over-production (69). Blunting ROS activity by intravenous infusion of ascorbic acid resulted in down-regulation of NOX2 together with TxB₂ reduction (69), thus providing indirect evidence of a NOX2 role in post-PCI TxB₂ over-production (69).

Obesity is characterised by an increased risk of atherosclerotic complication along with platelet TxB₂ over-production and enhanced oxidative stress as assessed by increased urinary excretion of 8-iso-PGF2α (72). Obese patients following a low calorie diet lost weight and reduced formation of TxB₂ and isoprostanes suggesting an interplay between oxidative stress and platelet activation (72). In accordance with these findings obese children and adults have been shown to exhibit platelet NOX2 up-regulation at the same time with platelet isoprostane over-production and platelet activation (73, 74).

Type II diabetes is a clinical setting characterised by accelerated atherosclerosis and platelet activation as indicated by increased platelet and urinary excretion of 11-dehydro-TxB2 and enhanced circulating biomarkers of platelet activation such as soluble CD40L and P-selectin (70, 75, 76). In addition to platelet TxB2 over-production, patients with diabetes had enhanced platelet oxidative stress together with enhanced isoprostane formation (70). A significant correlation was also found between platelet isoprostane formation and NOX2 regulation thus suggesting a role for NOX2 in eliciting platelet isoprostane over-production and platelet activation (70). This finding has been suggested to be implicated in impaired response to aspirin (aspirin resistance) as inhibition of TxA2 is counterbalanced by platelet isoprostanes over-production (70). This hypothesis has been partly supported by a pilot study with statin, which was administered on top of aspirin in a short-term study performed in patients with type II diabetes mellitus (77). The results showed that while aspirin alone was associated with NOX2 up-regulation and isoprostane over-production, addition of atorvastatin was able to counteract this phenomenon by down-regulating platelet NOX2 activation and isoprostane formation simultaneously reducing platelet activation.

Together these clinical studies provide indirect evidence of an interplay between platelet NOX2 and platelet activation but a cause-effect relationship needs to be confirmed by a pharmacological study with drugs directly affecting the enzyme.

Future perspectives

The data here reported suggests a role for ROS in activating platelet function with several mechanisms including NO inactivation, calcium mobilisation and isoprostane formation. In this context the role of NOX seems to be relevant as studies in platelets lacking NOX or in platelets treated with an inhibitor of NOX provided support to the central role of the enzyme in promoting ROS formation and eventually platelet activation. Among the NOX isoforms, NOX2 seems to have a prominent role as demonstrated by the almost complete absence of platelet ROS in patients with hereditary deficiency of this catalytic sub-unit. It is not possible, however, to exclude that other NOX isoforms may also contribute to platelet ROS formation and finally platelet activation. Thus, in patients with NOX2 hereditary deficiency platelet ROS production was still detectable even if in a small amount (4, 20). This issue has been recently addressed by Dayal et al., who investigated if other NOX isoforms may be detectable in platelets, but failed to show NOX1 or NOX4 (3). Further studies are, however, necessary to substantiate these results and to see if platelets may express other NOX isoforms.

Together the data herewith reported would suggest that down-regulation of platelet NOX may represent a novel option to inhibit platelet aggregation. However, a crucial issue is the possibility to down-regulate the enzyme without seriously interfering with the innate immune system. Thus, extension to which NOX may be reduced without interfering with the activity of innate immune system is a critical issue that should be taken into account. Some interesting clues on how developing a drug which inhibits platelet activation without interfering with innate immune system has been provided by a study showing that female carriers of NOX2 hereditary deficiency had less atherosclerotic burden compared to controls (78). Of note these females were not affected by serious infections despite that some of them suffered from immunologic diseases (78). This finding would imply that up to 50% NOX2 activity lowering may be relatively well tolerated without severe consequence for infectious disease defence.

Inhibition of cytosolic NOX sub-units is another potentially interesting therapeutic approach. In this context apocynin may be of interest because it inhibits the activity of p47phox. Of note, the hereditary deficiency of p47phox is characterised by a milder clinical course and longer survival (79). However, study exploring the anti-platelet effect of apocynin on platelet aggregation in human is still lacking.

In conclusion there is compelling evidence that platelets possess all the NOX2 armamentarium to produce ROS via this enzymatic pathway and in turn to elicit platelet aggregation. Further study is necessary to explore if platelets possess other NOX isoforms and their contribution to platelet activation. Human study is necessary to establish if inhibition of NOX is actually a worthwhile novel approach to inhibit platelet activation in patients at risk of atherothrombosis.

Conflicts of interest

None declared.

References


5. Pignatelli P, Carnevale R, Pastori D, et al. Immediate antioxidant and antitrau-


nulomatous disease in a child with a homozygous 36-bp deletion in the CYBA gene (A22 (0)) leading to the activation of a cryptic splice site in intron 4. Human Genetics 2002; 110: 444-450.


17. Li JM, Shah AM. Intracellular localisation and preassembly of the NADPH ox-


20. Pignatelli P, Sangivigni V, Lentì L, et al. gp91phox-dependent expression of pla-


24. Kawahara T, Kitohima M, Kuwano Y, et al. Helicobacter pylori lipopolysacchar-