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Review

Nox-2 up-regulation and platelet activation: Novel insights

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ABSTRACT

Platelet activation is a key step in the onset of cardiovascular complications in patients affected by systemic atherosclerosis. Among other mechanisms, oxidative stress seems to play a crucial role in platelet activation. Reactive Oxidant Species (ROS) including O_2^-, OH^- or H_2O_2 act as second messenger to activate platelets via (1) calcium mobilization, (2) nitric oxide inactivation and (3) through the interaction with arachidonic acid to give formation of isoprostanes. One important source of ROS is represented by platelet NADPH oxidase. Growing data from experimental and clinical studies provide evidence that Nox2, the catalytic core of the NADPH oxidase system, is implicated in platelet activation. Accordingly, an impaired platelet activation has been described in patients with genetically determined Nox2 deficiency. Moreover, platelets added with specific inhibitors of Nox2 revealed impaired platelet activation, along with ROS down-production. Similar results were seen in animals treated with apocynin, a Nox inhibitor, showed reduced platelet adhesion and atherosclerotic plaque. A significant association between Nox2 and platelet activation has been detected in patients with atherosclerotic diseases. The observed up-regulation of Nox2 with subsequent isoprostanes over-production in patients with cardiovascular diseases suggests the need to explore the potential benefit of targeting Nox2 as part of a holist anti-atherothrombotic strategy in patients with systemic atherosclerosis.

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1. Introduction

Platelets play a crucial role in the vascular homeostasis and in the onset of thrombotic complications in different atherosclerotic diseases [1]. Several physiologic and pathologic factors are able to induce platelet activation [2]. Among others, oxidative stress has been proposed as a regulator of platelet function, representing a common feature of many cardiovascular diseases. In particular NADPH oxidase seems to have a prominent role in atherosclerosis, being one of the most important enzymatic systems involved in Reactive Oxidant Species (ROS) formation [3,4].

ROS are chemically unstable compounds, rapidly reacting with other molecules inducing formation of oxidized products such as oxidized LDL and peroxynitrite [5]. Physiologically, ROS act as second messengers behaving as intracellular signals for cell activation [5]. There are different enzymatic pathways involved in the formation of ROS, among them, NADPH oxidase (Nox), myeloperoxidase, xanthine oxidase and uncoupled nitric oxide. In particular,

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Table 1
Isoforms and localizations of Nox.

Isoform	Localization	Subunits/mechanism of activation	Product
Nox1	Endothelial cells, vascular smooth muscles cells, hepatic stellate cells	$p22^{phox}$, NoxO1, $p67^{phox}$, NoxA1, Rac	Superoxide anion
Nox2	Endothelial cells, vascular smooth muscles cells, macrophages/Kupffer cells, platelets, fibroblasts	$p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, Rac	Superoxide anion
Nox4	Endothelial cells, vascular smooth muscles cells and fibroblasts	$p22^{phox}$	Hydrogen peroxide
Nox5	Endothelial cells, vascular smooth muscles cells	Calcium	Superoxide anion

NoxO1: Nox-organizing protein 1; NoxA1: Nox-activating protein 1.

Nox-related ROS production seems to facilitate platelet activation [6–8]. Thus, upon stimulation by common agonists, platelets produce ROS, such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). Both O_2^- and H_2O_2 contribute to the amplification of platelet aggregation [9].

The knowledge of mechanisms underlying ROS formation and exploring potential therapeutic approach modulating ROS formation may be helpful in the setting of athero-thrombosis.

The purpose of this review is to summarize available evidence on (1) data supporting the role of Nox in platelet ROS formation and platelet activation; (2) atherosclerotic diseases in which Nox up-regulation is implicated; (3) interventional trials with specific and non-specific inhibitors of Nox, including statins, polyphenols, apocynin and more recent evidence on some nutrients, such as olive oil.

2. NADPH-oxidase: structure and function

NADPH oxidase is a complex enzymatic system responsible for the generation of ROS [10]. This enzyme is able to transfer electron, using oxygen as electron acceptor and the product of the reaction is the superoxide anion (O_2^-). Different forms and localizations of the NADPH oxidase have been described [4] (Table 1). NADPH oxidase was initially described in phagocytic cells where, upon activation, NADPH oxidase produces extracellular superoxide, fundamental in host defense against microbial infections [11,12]. Data on NADPH oxidase function were studied in patients with genetically determined deficiency of different sub-units of this enzymatic system, referred to as Chronic Granulomatous Disease (CGD). Patients affected by CGD have a suppression of ROS production mediated by Nox2 activation, resulting in an impaired capacity of white cells response to bacterial infections, which seriously complicate the course of disease [11,12].

Specifically, we can distinguish between a phagocytic and non-phagocytic form of NADPH oxidase. The main difference between these two forms is related to the homologues replacing classical components [13]. The catalytic $gp91^{phox}$/Nox2 is typical of the phagocytic NADPH oxidase, while non-phagocytic form of NADPH oxidase discloses some homologues of $gp91^{phox}$/Nox2, including Nox1, Nox3, Nox4, Nox5, DUOX1/2. There are also homologues to the cytoplasmic components $p47^{phox}$ and $p67^{phox}$, namely NoxO1 and NoxA1, respectively. The non-phagocytic form of NADPH oxidase is present in endothelial cells [14], cardiomyocytes [15], hematopoietic stem cells [16], platelets [17] and hepatic stellate cells [13].

The mechanism involved in Nox2 activation has been well described in phagocytic cells. Phagocytic $gp91^{phox}$/Nox2 forms a membrane-bound heterodimeric flavocytochrome b558, associated with the $gp22^{phox}$ subunit. Activation of Nox2 requires the translocation of some cytosolic factors, namely $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and RAC to the membrane complex. Phosphorylated $p47^{phox}$ interacts with $p22^{phox}$ [18,19] and coordinates the translocation of other subunits ("organizer subunit"). Thus, $p47^{phox}$ takes the "activator subunit" $p67^{phox}$ into contact with Nox2 along with the small subunit $p40^{phox}$ [20]. Finally, the GTPase RAC interacts directly with Nox2 [21], and through the $p67^{phox}$.

Nox2 is also the only isoform of NADPH oxidase present in platelets. The organization of Nox2 system in platelets was described by Seno and colleagues that identified the presence of the $p22^{phox}$ membrane and $p47^{phox}$ cytosolic subunits [22]. Subsequently, we demonstrated the presence of the catalytic sub-unit $gp91^{phox}$ on platelet surface [7]. Nox2 activation in platelets also requires $p47^{phox}$, $p67^{phox}$ and RAC [7,17,22]. Activation of platelet Nox is crucial for O_2^- production, as shown by its almost complete suppression in patients with CGD [17]. These data provided insights on the role of platelets on the production of ROS and eventually on innate immune system.

The non-phagocytic isoforms of Nox are structurally different from the phagocytic one, requiring different mechanisms of activation. The Nox1 isoform has 60% of sequence identity with Nox2 [23]. Nox1 is present in vascular smooth muscle cells [24,25] and endothelial cells [26]. Similarly to Nox2, Nox1-mediated ROS production requires the $p22^{phox}$ and cytosolic subunits [27,28] named NoxO1 (Nox organizer 1) and NoxA1 (Nox activator 1), similar to $p47^{phox}$ and $p67^{phox}$, respectively. Nevertheless, Nox1 is also able to use the $p47^{phox}$ and $p67^{phox}$ subunits [23] and the GTPase RAC for its regulation [29].

Endothelial cells [30] and smooth muscle cells [31] also express the Nox4 isoform, along with fibroblasts [32]. Nox4 produces hydrogen peroxide, rather than superoxide, by a specific third extra-cytosolic loop that is not present in Nox1 and Nox2 [33,34]. Also Nox4 requires the $p22^{phox}$ subunit [28], but active Nox4 does not require cytosolic subunits or cell stimulation [33,35,36]. The last described Nox is Nox5 that is expressed in vascular smooth muscle cells [37]. Activation of Nox5 is mediated by a rise in the cytoplasmic calcium concentration that is bound by a specific calcium-binding domain on Nox5 [38], without cytosolic subunits.

3. ROS and platelet activation

Nox-derived oxidative stress significantly affects platelet function. The platelet O_2^- formation related to Nox activation enhances platelet aggregation and platelet-dependent thrombosis [39,40]. Accordingly, we observed a reduction of agonist-induced platelet aggregation in subjects with CGD, supporting the role of O_2^- in favoring platelet activation [7].

Nox-derived O_2^- may affect platelet activation via PLA_2-dependent arachidonic acid (AA) release from platelet membrane (Fig. 1). When generated, O_2^- is converted by the superoxide-dismutase (SOD) to H_2O_2, a chemically stable compound [41]. Platelet O_2^- act as second messenger to stimulate H_2O_2 and F2-isoprostanes generation or by interfering with nitric oxide (NO) activity/biosynthesis [7]. Thus, burst of H_2O_2 is associated with collagen-induced platelet aggregation, which activates platelets through intracellular calcium mobilization [41]. This causes an increased release of AA from platelet membrane, thromboxane A_2 and PLC up-regulation [41].

The crucial role for H_2O_2 in eliciting platelet activation has been recently confirmed by Dayal and colleagues in an in vivo