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Review Article

Organizers and activators: Cytosolic Nox proteins impacting on vascular function

Katrin Schröder[a,*], Norbert Weissmann[b], Ralf P. Brandes[a]

[a] Institut für Kardiovaskuläre Physiologie, Goethe-Universität Frankfurt, Frankfurt, Germany
[b] Excellence Cluster Cardiopulmonary System, University of Giessen and Marburg Lung Center (UGMLC), Member of the German Center for Lung Research (DZL), Justus-Liebig-University, 35392 Giessen, Germany

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ABSTRACT

NADPH oxidases of the Nox family are important enzymatic sources of reactive oxygen species (ROS) in the cardiovascular system. Of the 7 members of the Nox family, at least three depend for their activation on specific cytosolic proteins. These are p47phox and its homologue NoxO1 and p67phox and its homologue NoxA1. Also the Rho-GTPase Rac is important but as this protein has many additional functions, it will not be covered here. The Nox1 enzyme is preferentially activated by the combination of NoxO1 with NoxA1, whereas Nox2 gains highest activity with p47phox together with p67phox. As p47phox, different to NoxO1 contains an auto inhibitory region it has to be phosphorylated prior to complex formation. In the cardio-vascular system, all cytosolic Nox proteins are expressed but the evidence for their contribution to ROS production is not well established. Most data have been collected for p47phox, whereas NoxA1 has basically not yet been studied. In this article the specific aspects of cytosolic Nox proteins in the cardiovascular system with respect to Nox activation, their expression and their importance will be reviewed. Finally, it will be discussed whether cytosolic Nox proteins are suitable pharmacological targets to tamper with vascular ROS production.

1. Introduction

Nox NADPH oxidases are a family of enzymes with the sole function to produce reactive oxygen species (ROS). The ROS production by Nox enzymes is controlled on multiple levels like abundance of the enzymatically active Nox complex, substrate availability but also complex mechanisms of activation of some of the Nox enzymes [1]. Most review articles and original publications focus on the large catalytically active Nox enzyme acknowledging that this is certainly a rate limiting element for ROS production. Nevertheless, 3 of the 7 Nox enzymes are basically inactive under resting conditions and have to be activated by cytosolic proteins. The cytosolic proteins are controlled on the expression level, are subjected to regulation by signal transduction and can be limiting factors, too. Moreover, it is thought that these proteins have additional, so far underappreciated functions beyond their interaction with the catalytically active membrane-bound Nox subunit. Acknowledging this aspect, the present work will focus on the importance of the cytosolic Nox protein in the vascular system with respect to their impact on Nox activation, ROS production and cardiovascular physiology and pathology.

2. The Nox family of NADPH oxidases

2.1. The Nox homologues

The family of NADPH oxidases consists of 7 members: Nox1-5, as well as Duox1 and Duox2 [2]. Although all Nox enzymes produce ROS, they differ in activity and type of ROS generated. Nox1, Nox2, Nox3 and Nox5 produce primarily $\bullet O_2^-$, which subsequently may dismutate into H_2O_2, whereas Nox4, Duox1 and Duox 2 directly produce H_2O_2. Nox-derived ROS are required for host defense [2] and chemical modification of structures during compound synthesis of the body, like the thyroid hormone [3]. ROS produced by NADPH oxidases also act as second messengers and modulate protein activity and half-life of nitric oxide (NO) [4]. Whereas the latter function is mediated by $\bullet O_2^-$, H_2O_2 mediates the majority of the other functions of Nox-derived ROS [5]. H_2O_2 is a substrate for peroxidases and thiol oxidation in proteins is directly or indirectly mediated by H_2O_2.

Abbreviations: AIR, Autoinhibitory region; cAMP, Cyclic adenosine mono-phosphate; HUVEC, Human umbilical vein endothelial cells; LPS, Lipopolysaccharide; PI3K, Phosphatidyl inosite 3-kinase; PKA, Protein kinase A; PKC, Protein kinase C; PMN, Polymorph-nuclear neutrophils; ROS, Reactive oxygen species; SH3, Src homology 3 domain; TLR, Toll-like receptor; TGF, Transforming growth factor; TNF, Tumor necrosis factor; VSMC, Vascular smooth muscle cells

* Correspondence to: Institut für Kardiovaskuläre Physiologie, Fachbereich Medizin der Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany.
E-mail address: Schroeder@vrc.uni-frankfurt.de (K. Schröder).

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Functionally, Nox-derived signaling in the vascular system can be detrimental as well as protective. Physiological amounts of H_2O_2 maintain endothelial integrity and stimulate anti-inflammatory systems [6], overproduction of H_2O_2, however, can be pro-inflammatory, induces oxidative stress and endothelial dysfunction [7–9]. Very few reports suggest a beneficial function of $•O_2^-$ [10,11]. This is probably the consequence of the fact that, in contrast to H_2O_2, $•O_2^-$ reacts with NO on diffusion distance to form peroxynitrite ($ONOO^-$). Thus, $•O_2^-$ not only limits NO level but also promotes the formation of the highly oxidizing $ONOO^-$–. By that mechanism, overproduction of $•O_2^-$ is rather detrimental to endothelial function [4,12], provides a pro-inflammatory stimulus and mediates uncoupling of endothelial nitric oxidase synthase (eNOS) [13]. Nevertheless, once dismutated to H_2O_2, also Nox-derived $•O_2^-$ at low level, might be important for the vascular homeostasis [14].

All NADPH oxidases are multi-subunit enzymes, where the subunits need to form a complex in order to allow the proper function of the NADPH oxidase resulting in the formation of the ROS mentioned above [2]. Nox5 is an exception as its activity depends on oligomerization of the large catalytically active Nox protein [15], whereas all other Nox enzymes are heterodimers. Nox5 is calcium-dependent and generates primarily $•O_2^-$ [16]. As the enzyme is, to our current knowledge, not dependent on cytosolic proteins, it will not be further covered in this article.

The other calcium-dependent group of NADPH oxidases is the Duox family. Duox1 and 2 are located at the apical pole of epithelial cells where they produce H_2O_2 and entertain oxidation processes through peroxidases like the oxidation of iodide in the thyroid gland or host defense in the lung epithelium. Duox1 and 2 require the membrane bound subunits DuoxA1 and DuoxA2, respectively, for their proper function. For details, the reader is referred to an excellent review of this system [17]. In the vascular system expression of Duox1 and 2 appears to be insignificantly low and a role for these enzymes in the vasculature has not been reported. Therefore, the Duox family will not be a focus of this article.

The remaining NADPH oxidases 1–4 require a membrane bound subunit, p22phox, for stabilization of the Nox subunit [18,19]. For Nox4 no further subunit is required which leads to the constitutive activity of the enzyme [16,20,21]. Interestingly Nox4, like the Duox-enzymes, appears to directly produce H_2O_2 which is a function of an extended E-loop of the enzyme [22–24]. As Nox4, the remaining three NADPH oxidases Nox1-3 are dependent on the complex of the large catalytic Nox subunits and p22phox [21,25]. However, to activate them, additional cytosolic subunits have to translocate to this complex.

2.2. Cytosolic Nox proteins

The cytosolic proteins of concern, which are also the focus of this article, are p47phox, p67phox, NoxA1 and NoxO1 [2]. To some extent also p40phox of which the function is somewhat unclear will be discussed. In addition to these proteins, the small GTPase Rac is involved in Nox activation. Rac2 is required for activation of leukocytes NADPH oxidase Nox2 as has recently been reviewed excellently by a pioneer in the field, Edgar Pick [26]. In other cells, Rac1 appears to play a similar role for the Nox activation at least of Nox1 and Nox2. Dependency of Nox4 on Rac is not well established and whether Nox3 is activated by Rac is under debate [27,28]. As the Rho-GTPase Rac1 has numerous additional functions, a comprehensive presentation of the functions of this important protein would go beyond the focus of this article. The reader is referred to recent specific review articles [29,30].

In vascular cells, the NADPH oxidases Nox1, 2, 4, and 5 are expressed [31]. As pointed out above, out of those only Nox1 and Nox2 require cytosolic subunits. In the sections below, first the molecular mechanism of the function of these cytosolic subunits will be reviewed briefly. In the subsequent section, the evidence of the importance of the cytosolic subunits in the vascular system will be presented. Finally, aspects of pharmacological exploitation of the interaction of the cytosolic subunits will be discussed.

3. Nox activation by cytosolic subunits

3.1. Modes of activation of Nox1, Nox2 and Nox3

On the basis of maximal activation and expression similarities, the cytosolic subunits activating Nox2 differ from that to activate Nox1. In the classic model, the leukocyte, Nox2 is activated by p67phox (and Rac2) and the complex is tethered to Nox2 by p47phox, which acts as adapter. Control of activity is exerted on multiple levels but most importantly through an auto-inhibitory region of p47phox, which has to be displaced by phosphorylation to allow protein-protein interactions with other proteins (details below). In the case of Nox1, the activator function of p67phox is carried out by NoxA1 (Nox-Activator1) and the organizing function of p47phox is fulfilled by NoxO1 (Nox-Organizer1). Different to p47phox, NoxO1 does not contain the auto-inhibitor region and thus the Nox1-NoxO1-NoxA1-complex has high basal activity (for a detailed review see [1]).

In overexpression systems, alternative complex compositions (i.e. Nox1 with the phox proteins or different combinations of cytosolic organizers and activators) have been studied but none of those exhibited a comparably high activity as the natural complex [32–34]. Nevertheless, it is conceivable that in cells of the vascular system, where all cytosolic proteins are expressed, albeit to a low level, alternatively composed Nox complexes are formed.

Different to Nox1 and Nox2, the only cytosolic protein required to activate Nox3 is NoxO1 [25,33,35] Low activity can be obtained with the combination of Nox3 with p67phox, which is further increased upon addition of p47phox. P47phox or NoxA1, however, are not able to activate Nox3 and NoxA1 appears to be totally unimportant for the activity of human Nox3 [25].

3.2. Detailed aspects of the cytosolic proteins

3.2.1. The organizer protein p47phox

p47phox (gene name neutrophil cytosolic factor1 - NCF1) is a protein consisting of 390 amino acids with a molecular mass of 44.7 kDa. P47phox was first discovered in 1989 by two independent groups [36,37]. Amino acids 4–121 represent a PX (phox homology) domain for membrane interaction while amino acids 159–214 and 229–284 represent two SH3 domains for protein-protein-interaction. At the C-terminus, p47phox contains a basic charge and one proline-proline-arginine-arginine-containing region (amino acids 363–368) [38] which acts as auto-inhibitory region (AIR). This interacts with the N-terminal SH3 domain in order to keep p47phox in a resting state [39]. In case of activation, the AIR is displaced and the tandem SH3 domains bind to p22phox. Deletion of the AIR renders p47phox constitutively active [25,40]. The critical step in the activation of p47phox-dependent ROS production is the phosphorylation of p47phox by protein kinase C (PKC). Alternative kinases to PKC are protein kinase B (AKT) and p21-activated kinase (PAK-1). All these phosphorylate several serine residues with Ser379 portably being the most important, at least in murine leukocytes [41]. As a consequence of this phosphorylation, the AIR is displaced and p47phox can interact with p22phox, the membrane and p67phox. Beside classic signal transduction including phosphorylation, probably mitochondrial ROS can trigger the translocation of p47phox [42]. Together with Rac, the essential function of p47phox is to tether the activator subunit p67phox to the Nox2 protein to allow electron flux from NADPH through the enzyme to molecular oxygen [25] (Figs. 1 and 2).

3.2.2. The organizer protein NoxO1

NoxO1 was discovered in 2003 as structurally and functional wise

similar to p47phox with a predominant expression in the epithelium but also the inner ear [32,35]. NoxO1 has a molecular mass of 39 kDa and displays 25% sequence identity to p47phox. Similar to p47phox, NoxO1 has two SH3 domains as well as the C-terminal proline-rich region which enables p47phox to interact with p67phox. The isolated tandem SH3 domains of NoxO1 bind to p22phox with high affinity and this is severely inhibited in the presence of the C-terminal tail of NoxO1, suggesting that this region competes for binding to p22phox and thereby contributes to the regulation of superoxide production to some extent [43]. Importantly, NoxO1 lacks the proline-proline-arginine-arginine-containing region of p47phox, which is involved in auto-inhibition, it also does not contain the adjacent serine phosphorylation sites, which control the configuration of the AIR [32]. Consequently, NoxO1 due to the lack of those regulatory elements constitutively interacts with the activator protein NoxA1. NoxO1 also contains a PX-homology domain, which targets proteins to the phosphoinositide groups of membranes where it binds to the phosphatidylinositol (PtdIns) lipids PtdIns 3,5-P2, PtdIns 5-P, and PtdIns 4-P [44]. In p47phox, the domain is also only exposed upon release of the AIR. As NoxO1 lacks this structure, NoxO1 constitutively interacts with the membrane (Figs. 1–3).

NoxO1 is encoded by eight exons with seven intervening introns. Four splice forms are described (α,β,δ,γ) and generated by alternative splicing of exon 3, resulting in forms that differ by 3 nucleotides in the 5′ end of exon 3 and/or 15 nucleotides in the 3′ end of exon 3. Those splice differences are in the PX domain, implying an effect on membrane binding, which has not been proofed so far. However, NoxO1β seems to be the most important splice variant of NoxO1 for Nox mediated ROS formation [45].

Importantly, the constitutive activity of NoxO1 can be modulated.

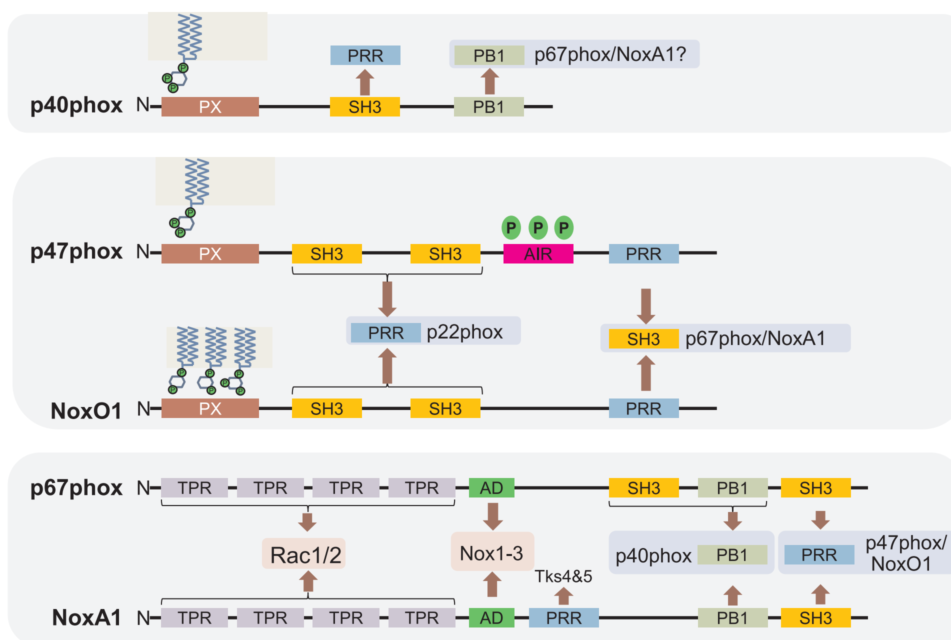

Fig. 1. Scheme of domains of NADPH oxidases subunits. AD - activation domains, AIR - autoinhibitory region that is inactivated upon phosphorylation, PB1 - phox and Bem1 domain, PPR - proline rich region, PX - phox homology domains that bind to PtdIns-phosphates in the membrane, SH3 - Src homology 3 domains, TPR - tetricopeptide repeat domains that interact with Rac.

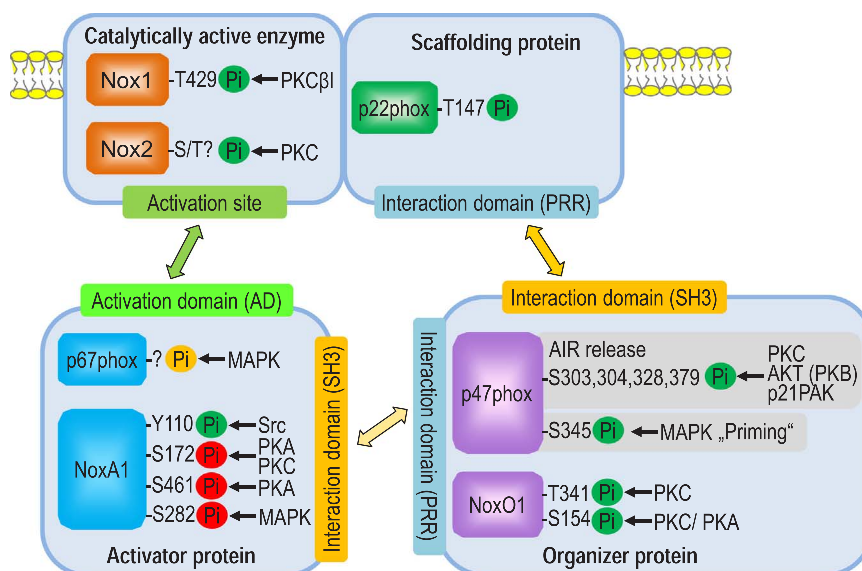

Fig. 2. Scheme of phosphorylation sites of NADPH oxidases Nox1 and 2 as well as subunits of the complex.

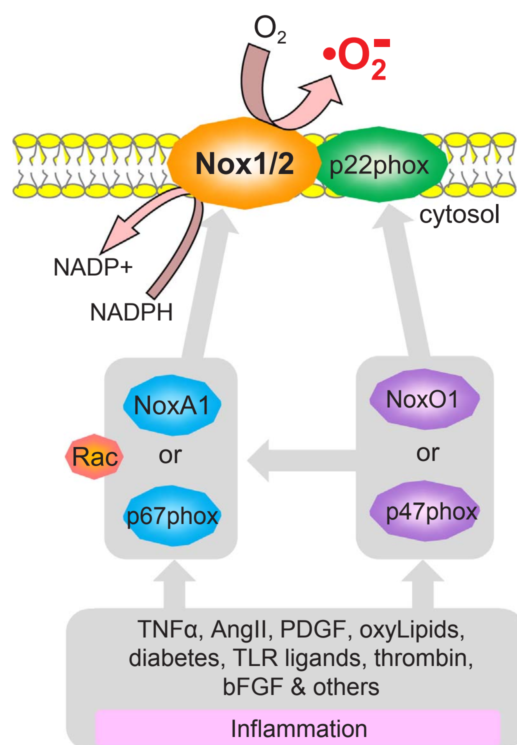

Fig. 3. Scheme of induction of the cytosolic subunits of NADPH oxidases in the

NoxO1 phosphorylation by PKC at Ser154 doubles its binding ability to NoxA1, which in turn acts as a molecular switch, allowing optimal interaction of NoxO1 with p22phox [46]. Further reports indicate that NoxO1 can be phosphorylated by PKA upon treatment with 8-bromo-cAMP or forskolin at Ser154 and upon PMA treatment by PKC at Thr341 with both phosphorylations increasing the activity of Nox1 mediated ROS formation [47] (Figs. 1 and 2).

3.2.3. The activator protein p67phox

p67phox (gene name neutrophil cytosolic factor1 - NCF2) is a protein with 526-amino-acids and a molecular mass of 59.8 kDa. It consists of a four N-terminal TPR (tetratricopeptide repeat) motif-containing domains, one proline rich region and two SH3 domains with a PB1 (Phox and Bem1) domain in between. The TPR domain is responsible for the interaction with Rac [48]. Phospho peptide mapping of p67phox indicated that ERK1/2 and p38MAPK phosphorylate different subgroups of peptides. The major phosphorylation target site of ERK1/2 is the N-terminal fragment (1–243), while the major phosphorylation target sites of p38MAPK are located in the C-terminal fragment (244–526). Further an additional peptide, which was not phosphorylated in the intact protein, appeared to be phosphorylated in the isolated C-terminal fragment (aa 244–526), indicating that this site may become accessible after a conformational change of the protein [49]. Via its PB1 domain, p67phox mediates the binding to p40phox [50]. p47phox associates with p67phox via its C-terminal PRR and the second SH3 domain of p67phox [51]. Interestingly the PRR of p47phox is surrounded by a number of serine residues (Ser359, Ser370 and Ser379), which are known to become phosphorylated during NADPH oxidase activation, which significantly weakens the interaction with p67phox, raising the possibility that there is a basal level of phosphorylation that could prevent the interaction between p47phox and p67phox and that a phosphatase may be involved in the proper activation of the Nox protein [38]. Finally, p67phox contains an 'activation domain' (amino acids 199–210) that is absolutely required for $\cdot O_2^-$ production in a reconstituted cell-free system and may bind directly to Nox2 [52] (Figs. 1 and 2).

3.2.4. The activator protein NoxA1

NoxA1 has a molecular mass of 49 kDa and displays 30% sequence identity with p67phox. It contains tetratricopeptide repeats, which are important for Rac binding in p67phox, the activator domain, which interacts with Nox2 and it conserved the C-terminal SH3 domain of p67phox. NoxA1 has a C-terminal hydrophobic stretch, which may represent a transmembrane tail [32]. Nevertheless, plasma membrane targeting of NoxA1 depends on NoxO1, through tail-to-tail interactions between these proteins. Under physiological expression conditions, activation of Nox1 by NoxA1 involves Rac1 [28] but Rac binding to the Nox1 complex does not require NoxA1 or NoxO1 [53]. Others found that Rac1 binds to the activation domain of NoxA1, which contains 4 units of tetratricopeptide repeat motifs. Nevertheless, a mutant NoxA1 carrying the substitution of Ala for Val-205 in the activation domain, which is expected to undergo a conformational change upon Rac-binding, fully localizes to the membrane but fails to activate Nox1 [54].

NoxA1 contains an N-terminal proline rich region that is missing in p67phox. This PRR allows the binding of the c-Src substrate proteins Tks4 and Tks5, which supports NoxA1s binding to Nox1 and thereby Nox1-dependent ROS generation [55]. NoxA1 can be phosphorylated in a MAPK (Erk1/2 and p38) and protein kinases C and A -dependent manner. In fact Ser282 represents the target of MAP kinases while Ser172 is targeted by PKC and Protein kinase A (PKA). In an over-expression model using HEK293 cells, phosphorylation of these sites was observed even in the absence of stimuli and limited constitutive Nox1 activity due to a decreased binding of NoxA1 to Nox1 and Rac1 [56]. Others identified Ser172 and Ser461 of NoxA1 as phosphorylation sites for PKA. Upon phosphorylation NoxA1 complexes with 14-3-3zeta which further depresses Nox1 complex formation and ROS production [23] (Figs. 1 and 2).

3.2.5. p40phox

Neutrophil cytosol factor 4 (NCF4) or p40phox is predominantly expressed in bone marrow-derived cells: neutrophils, monocytes, basophils, eosinophils, mast cells, megakaryocytes, B and T cells [57]. It is a 40 kDa protein which complexes with p67phox and p47phox and whose primary association appeared to be with p67phox, also in resting state of phagocytes. The level of this protein were reduced in patients with CGD lacking p67phox, indicating, that p40phox may be stabilized by p67phox [58]. p40phox consists of 339 amino acids and has a PX domain (aa 19–140), a SH3 domain (aa 175–225) and a PB1 domain (aa 285–306) [59]. The true function of the protein even in leukocytes is somewhat unclear. It however appears that p40phox is an upstream mediator of Nox2 activation in leukocytes rather than an intricate component of the Nox2 complex [57]. In the reconstitution assay, p40phox does not promote the complex activity [60] and the disease phenotype of p40phox-deficient patients is milder than that of p47phox deficiency [57] (Figs. 1 and 2).

4. Cytosolic Nox proteins in the vascular redox-context

4.1. Protein expression

Clearly protein expression is a limiting factor for enzyme activity. This is best documented for the catalytically active Nox enzymes, were a link between protein abundance and ROS production is obvious [61]. For the cytosolic Nox proteins, tissue specific expression has been documented and numerous publications presented mRNA expression data. On the protein level, the situation, however, is less clear owning that lack of specific antibodies with sufficient sensitivity and an insufficient control of Western blots and immunohistochemistry by loss of expression techniques such as siRNA or genetic knockout. Moreover, the expression of the cytosolic subunits is not constitutive as dedifferentiation in cell culture and many other factors like inflammation, hypoxia and growth factors impact on their expression.

An exclusive focus on protein expression suggests that high expres-

sion of p47phox and p67phox is found only in myeloid cells [2], whereas NoxO1 and NoxA1 are specifically enriched in epithelial cells of the gut and some specific other sites like testis and inner ear. The vascular system at large exhibits somewhat low expression of all these subunits but some of them are induced by inflammation (Fig. 3).

4.1.1. Expression of p47phox

By RT-PCR p47phox can be detected in the cells of the vascular system. On the protein level, the situation is less convincing. Numerous histologies and Western blots have been published but only few of them were controlled by siRNA or loading of samples from knockout mice. In cultured mouse aortic smooth muscle cells our lab failed to obtain a signal by Western blot and immuno fluorescence [62] and also another early report on p47phox, the protein was hardly detectable by immuno fluorescence [63]. In cultured murine endothelial cells, a specific predominantly cytoskeletal staining was observed, which was not present in p47phox knockout cells [64,65] and a distinct band was observed by Western blot which was also lost after knockout of the protein [66]. Another study in microvascular endothelial cells demonstrated that the specific band is subject to cytokine induction [67], but siRNA or knockout strategies were not applied to confirm specificity of the signal.

It is remarkable and also hard to understand that there are almost no reports on p47phox protein expression in studies comparing organs of WT and p47phox-/- mice. Numerous studies show p47phox immunohistochemistry or immunofluorescence but do not provide the knockout control. When knockout mice are used, deletion of p47phox usually leads to a substantial loss in ROS production but this is not linked to protein expression. To our knowledge, the only studies showing such p47phox Western blots, but also not immuno-histochemistry comes from S. Selemidis' group [68]. Potential explanations for this might be that even a few contaminating leukocytes are sufficient to generate the ROS signal or that p47phox protein expression is simply so low that it cannot be detected with standard technique. Western blot extract can easily be contaminated with leukocytes, too. Obviously, the reduction in ROS signal upon deletion of p47phox speaks for a functionally important expression of the protein. Nevertheless, it cannot be excluded that differences in the genetic background or epigenetic drifts might also contribute to this effect.

4.1.2. Expression of p67phox

As a component of the leukocyte NADPH oxidase, p67phox is expressed in leukocytes but was suggest to also be found in endothelial cells and in the aortic adventitial fibroblasts [69,70]. Importantly although p22phox as well as p47phox are expressed to the same extent in endothelial cells and leukocytes, p67phox and Nox2 are present at much lower levels (2.5% and 1%) [71]. Interestingly, p67phox expression by Western blotting was used to quantify adherent leukocytes on cultured endothelial cells [72]. In smooth muscle cells p67phox was also detected and found to be translocated to the membrane upon stimulation of the cells with Ang II [73]. We, however failed to detect p67phox in murine aortic smooth muscle cells and believe that it was replaced by NoxA1 (see below) [74]. Obviously, the criticism raised concerning p47phox can also be brought up for p67phox, where not even a knockout mouse has been published. Thus, it is unclear which of the published data is just an over-interpretation of an unspecific signal or consequence of leukocyte invasion into the vessel wall, as occurring in response to cytokines or angiotensin II.

Interesting data were reported for the kidney of Dahl salt sensitive rats: Under resting conditions p67phox was quasi undetectable but salt-induced hypertension increased the signal. Importantly, as the authors generated a knockout model, they also convincingly demonstrated that the p67phox protein band in Western blot was missing in the same samples of the knockout animals [75]. Unfortunately, immuno histochemistry was not carried out. Thus, it remains unclear whether the signal is consequence of renal induction of migration of leukocytes into the kidney. Moreover, p67phox expression has been documented primarily in epithelial cells. Thus, whether the signal reported in this study reflects vascular p67phox is questionable.

4.1.3. Expression data of p67phox and p47phox in response to stimulation

Taking into account these limitations and caveats, numerous studies suggest that p67phox abundance is increased in response to growth factors and cytokines. Most of these points also apply to p47phox and thus these reports on expression are listed here together. Although increased upon LPS treatment in total aorta by 6 fold p67phox and Nox2 did not directly contribute to endothelial dysfunction, as ROS scavengers were not able to reverse the impaired endothelial dysfunction induced by LPS [76]. Ang II infusion also increased the expression of NADPH oxidase components Nox2 to 3 fold and p67phox to 1.5 fold and simultaneous blockade of the AT1-R with lorsatan prevented that increase as well as the elevation in blood pressure. However, it again remained controversal, whether NADPH oxidase-derived ROS result from tissue resident macrophages, invading cells into the adventitia in response to inflammation or local induction in adventitial fibroblasts [77–79]. Interestingly high salt/Ang II induced upregulation of p67phox further enhanced in mice expressing reduced levels of adrenomedulin by an unknown mechanism [80]. The same result was found in a model of arterial intimal hyperplasia induced by cuff injury in rats. Importantly in that work the authors identified adventitial CD45 positive cells abundance to correlate with p67phox upregulation, which was less pronounced upon reduced adrenomedulin expression [81]. This might indicate that indeed inflammation is the main driver of p67phox upregulation and activation of ROS formation in several pathological models. In a model of aortic banding expression of p67phox together with p47phox and Nox2 was increased in the hypertensive zone above the band without specifying the exact localization of its expression and remained unchanged in none affected parts of the vessel [82]. Importantly p22phox, p67phox, and p47phox were significantly increased in patients with coronary artery disease and NADPH oxidase activity was increased by PKC and AngII [83]. Although inflammatory markers were not measured in the aortic banding nor in the human coronary arteries it is likely, that rather an inflammatory response and leukocyte infiltration is associated with upregulation of p67phox. Indeed, in inflammatory states such as atherosclerosis p67phox is upregulated in human carotid artery [84,85]. Accordingly in a study of human chronic heart failure upregulation of the inflammation marker C-reactive protein (CRP) was associated with an increased expression of p67phox in saphenous veins [86].

Besides inflammation also increased pressure load appears to induce the expression of p67phox. In a vein graft model which is associated with an increased pressure load of the vein, ROS formation was increased and p22phox as well as p67phox were upregulated in the transplant. However, the detailed localization of p67phox remains elusive, while p22phox was mainly upregulated in smooth muscle cells [87]. Accordingly in p67phox and Nox2 expression are elevated in high-flow over control arteries in obese Zucker rats, which was associated with flow induced remodeling [88]. In a model of pulmonary hypertension in fetal lambs p67phox was upregulated in pulmonary arteries, but not veins. There it contributes to vascular constriction and subsequent hypertension by the same NO dependent mechanism [89]. Indeed p67phox expression appears to be induced by a mechanosensitive mechanism. In swine p67phox expression is higher in pressure exposed vessels such as coronary artery and aorta over femoral and renal arteries. Additionally p67phox expression is always higher in the artery over the corresponding vein in pigs [90].

In porcine coronary arteries balloon-induced injury resulted in an upregulation of p47phox and p67phox in adventitial fibroblasts as shown by immunohistochemistry. Importantly fibroblasts proliferation was abrogated upon treatment of isolated cells with antioxidants [91].

In vitro treatment with the β1-blocker nebivolol but not atenolol or

metoprolol induced a dissociation of p67phox and Rac1, as well as an inhibition of NADPH oxidase activity in heart membranes from angiotensin II-infused rats, as well as in homogenates of Nox1 and cytosolic subunit-transfected and PMA-stimulated HEK293 cells. These findings indicate that nebivolol interferes with the assembly of NADPH oxidase [92]. Also in diabetes p22phox together with p47phox and p67phox appear to be upregulated and contribute to an increased superoxide formation resulting in a reduced bioavailability of NO [93]. Of importance the dipeptidylpeptidase-4 inhibitor linagliptin which is used to lower blood glucose levels in diabetes type 2 revealed positive effects against cardiac fibrosis in Dahl salt-sensitive hypertensive rats under high salt loading. There the treatment with the inhibitor reduced the expression of p67phox and p22phox and subsequently reduced the formation of ROS and cardiac fibrosis [94]. This effect however might be related to an anti-inflammatory effect of linagliptin as it may inhibit the activity of the glutaminyl cyclase iso-enzymes QC (QPCT) or isoQC (QPCTL) which is involved in CCL2 expression [95]. By inhibition of the QPCT linagliptin may prevent macrophage infiltration and act anti-inflammatory, which would explain the reduction in p67phox as this NADPH oxidase subunit is highly expressed in macrophages.

In conclusion, cardiovascular p67phox increases in response to growth factors and cytokines, but the cellular source of the protein is unclear.

4.1.4. Expression of NoxO1

The main expression sites of NoxO1 are colon and testis [2]. We detected vascular NoxO1 expression by immunohistochemistry. Importantly, in a recently generated knockout mice, the expression signal for NoxO1 was lost confirming the specificity of the signal detected in our lab on the basis of immunohistochemistry [96]. In cell culture experiments of bovine aortic endothelial cells, NoxO1 expression was reported to be induced by oscillatory flow. Importantly, siRNA against NoxO1 decreased the observed basal and stimulated signal confirming the specificity of this observation [97].

All other data on expression control either refer to mRNA expression or do not apply the same level of controls: NoxO1 expression was found in the endothelium of the rat basilar artery [98]. NoxO1 is also expressed in vascular smooth muscle cells and monocytes, probably via a HSP90 dependent pathway. There NoxO1 together with Nox1 may contribute to atherosclerosis development [99]. Other studies suggest that basal expression is very low and that the enzyme has to be induced for inflammatory cytokines or hypoxia [100,101]. In ob/ob mice the expression of NoxO1 (and NoxA1) in small mesenteric arteries was drastically increased, while expression of the Nox subunits remains low with a slide increase in Nox1 expression [102].

4.1.5. Expression of NoxA1

Data on knockout animals are not available for NoxA1. Using an antisense plasmid, we reported that NoxA1 substitutes for the lack of p67phox in mouse smooth muscle cells [74]. It was suggested that in endothelial cells NoxA1 contributes to Ang II and oxLDL induced ROS formation and oxLDL increased the expression of NoxA1 in endothelial cells [103]. In that study NoxA1 expression was significantly increased in aortas and atherosclerotic lesions of ApoE-/- mice compared with age-matched wild-type mice. Furthermore, in contrast to p67phox, immunoreactive NoxA1 was observed in the intimal and medial SMCs of human early carotid atherosclerotic lesions [104]. Collectively, these data suggest that NoxO1 and NoxA1 are induced by cytokines and growth factors. In smooth muscle cells, p67phox expression is very low and NoxA1 might replace this subunit. NoxO1, in contrast is expressed in endothelial cells under basal conditions and expression in the smooth muscle layer has not been studied sufficiently.

4.1.6. Expression of p40phox

Very little is known about p40phox, which is not a central aspect of Nox research. P40phox was reported to be expressed under basal conditions in endothelial cells [105]. Others also found p40phox in endothelial cells, but did not further characterize its function in those cells [106]. No knockout mouse data have been reported and siRNA studies are lacking to confirm the specificity of these observations.

4.2. Functional consequences

In the following paragraphs, the relevance of the individual cytosolic proteins to Nox-dependent ROS formation and signaling in the cardiovascular system will be reviewed (Fig. 4).

4.2.1. P47phox

Of all cytosolic subunits, p47phox is the best studied one. Mutations in p47phox are the second most common cause auf chronic granulomatous disease (CGD) and thus clinical studies are possible. The p47phox mouse was generated already in 1995 [107] and has been excessively studied whereas for the other proteins, knockout models have just recently become available.

Despite the fact that p47phox mutations result in CGD, its absence has been shown to improve vascular function in humans due to an increased nitric oxide availability [108,109]. Angiotensin II increased the formation of $\bullet O_2^-$ in wildtype but not in p47phox knock out mouse aortic endothelial cells [110]. In line with this p47phox-/- mice exhibited attenuated angiotensin II-induced hypertension [111] and angiotensin II-induced endothelial dysfunction [112]. In line with this, deletion of p47phox prevented aneurysm formation in models utilizing angiotensin II [113,114], but also in a cerebral aneurysm model [115]. Genetic deletion of p47phox also protected against myocardial infarction–induced cardiac dysfunction [110]. Also in other models of hypertension, like the DOCA-Salt model [13], deletion of p47phox was protective. Moreover, p47phox was required to increase endothelial permeability in response to cytokines [116].

Interestingly, whereas one study failed to detect an effect of p47phox knockout on basal blood pressure [117], two other groups report that genetic deletion of the protein resulted in basal hypertension [111,112], which was accompanied by an increase in basal vascular $\bullet O_2^-$ formation in one study [112]. The basal vascular dysfunction after deletion of p47phox was sensitive to inhibitors of the renin-angiotensin system and consequence of an activation of the renin-angiotensin-system [111]. The source of the increased basal ROS production in p47phox-/- mice has not well been studied. In general, deletion of the Nox2/p47phox-system leads to a systemic hyper-inflammatory phenotype [118] and several ROS producing systems are induced or activated by inflammation, like xanthine oxidase or mitochondrial ROS and eNOS

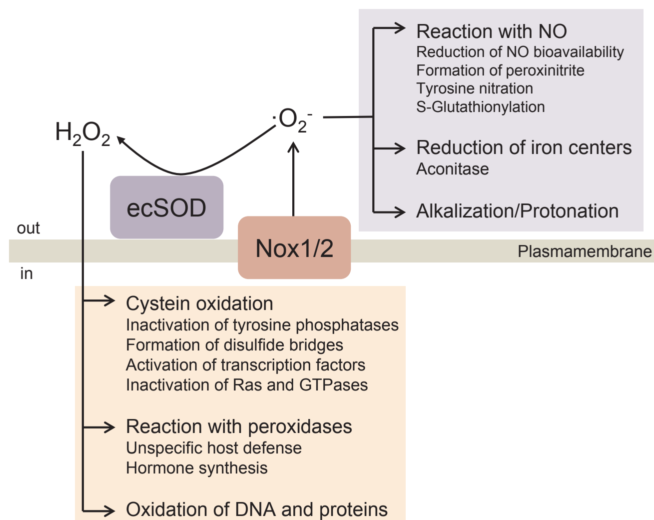

Fig. 4. Potential functions and consequences of Nox1 and 2 derived ROS formation.

uncoupling. Nevertheless, it is also attractive to speculate that deficiency of p47phox promotes the interaction of the Nox protein with NoxO1 (see below), which should increase basal ROS production. Nevertheless, basal vascular ROS production was increased in Nox1-Nox2-Nox4 triple knockout mice [119] which rather speaks for alternative sources of ROS.

In isolated vessel, p47phox knockout failed to modulate the response to angiotensin II, which might suggest that inflammatory cells mediate vascular effects of p47phox upon AngII treatment [64]. Indeed, it is well known that AngII leads to recruitment of myeloid cells and that deletion of monocytes prevents vascular effects of AngII [79]. In line with this, the mobilization of endothelial progenitor cells from the bone marrow is blunted in p47phox knock out mice as shown in a two-kidney one-clip model [120]. The aspect of myeloid versus vascular p47phox is somewhat difficult to dissect as conditional knockout mice for p47phox are not available. Bone marrow transplant experiments, however, suggested that the Nox-dependent effects on blood pressure are rather mediated by p47phox in immune cells than in vascular cells [121].

Nevertheless, there is also substantial cell cultured evidence that deletion of p47phox attenuates agonist-stimulated ROS production to growth factor and cytokines [14,62]. The basis of this discrepancy is somewhat unclear. Cultured vascular cells, compared to leukocytes, generate only very low amounts of ROS, which are, however sufficient to have strong effects on cellular signal transduction [5]. This is on one hand a consequence of both: an increase in the sensitivity and of the usually high level of growth factor stimuli used. Moreover, the state of differentiation in the body is very different from that in culture which may result in altered protein expression.

As mentioned above, p47phox was involved in the breakdown of endothelial barrier function in response to tumor necrosis factor α [116]. Interestingly, recently p47phox has been shown to mediate the opposite effect in response to angiopoietin. The barrier preserving function of this autacoid was dependent on p47phox in an LPS treatment model. p47phox facilitated the angiopoietin 1 mediated activation of Rac1 and inhibited LPS-induced activation of RhoA [122].

Atherosclerosis is a situation of increased vascular ROS production, which is also at least in parts mediated by p47phox [117,123]. Whether this also impacts on atherosclerosis development, however, is unclear. Some studies suggest that deletion of p47phox [123] or other Nox enzymes [124] reduce atherosclerosis development, others failed to observe this effect [117,125]. It is not known whether this discrepancy is consequence of the hyperinflammatory state present in Nox knockout mice, the rapid nature of atherosclerosis development in mouse model or the potential fact that NADPH oxidases might not be important for atherosclerosis development.

Collectively, these data suggest that p47phox is very important to mediate agonist-induced ROS production in the vasculature. Lack of p47phox attenuates angiotensin II- and cytokine mediated oxidative stress and its consequence. It, however, also increases the basal ROS production and leads to some basal dysfunction, which is mechanistically not understood.

4.2.2. NoxO1

Mice carrying mutations in the NoxO1 gene were already reported in 2006 [126] but to our knowledge these animals were never phenotyped in the cardio-vascular system. Our lab recently generated a conditional NoxO1 knockout mouse and observed that NoxO1 was important for basal endothelial $\bullet O_2^-$ production and signaling [96]. As a consequence of the NoxO1-induced ROS production, the α-secretase TACE (TNFα converting enzyme) was induced and activated which facilitated Notch signaling. Loss of NoxO1 resulted in attenuated release of the Notch intracellular domain with an increase in tip cell formation of the endothelium. NoxO1 knockout mice therefore exhibit increased angiogenesis [96].

To our knowledge, no other studies addressed the vascular function of NoxO1 on the basis of knock mouse models, but some additional observations were published with in vivo siRNA approaches. Although this technique has many pitfalls, the specific study supports our view that NoxO1 contributes to vascular $\bullet O_2^-$ production: In mice with STZ induced diabetes in vivo siRNA against NoxO1 in combination with Nox1 reduced uncoupling of eNOS [114]. This approach was as effective as the use of a p47phox-/- mouse in the same model, suggesting, that p47phox and NoxO1 in combination mediate ROS formation by Nox1, Nox2 or both that eventually contribute to eNOS uncoupling. In an in vitro approach of laminar and oscillatory shear stress NoxO1 has been shown to be involved in the uncoupling of eNOS together with Nox1, only in oscillatory shear stress [97], which complements earlier observations that endothelial cells induce Nox1 upon oscillator flow [127]. Interestingly, NoxO1 but not p47phox appeared to be a specific mediator or oscillator flow-induced endothelial dysfunction: Laminar flow exclusively induced the formation of the p47phox/ Nox2 complex, that promoted activation of eNOS by Ser1179 phosphorylation [97].

Collectively, still very little is known about NoxO1 regarding expression as well as function in the vascular system. The current data suggest that inflammation and cellular activation induce NoxO1, which then promotes $\bullet O_2^-$ formation and development of vascular dysfunction. That NoxO1 also has a positive physiological function in the vascular, as it does have in the inner ear, so far has not been reported.

4.2.3. p67phox

To our knowledge, data about p67phox knockout mice have not been published but a p67phox knockout rat was generated in the Dahl system by zinc-finger nuclease technique [75]. This approach resulted in reduced hypertension development and attenuated ROS formation. In subsequent studies of these animals it was observed that high-salt diet induced a loss of renal blood flow and glomerula filtration rat which was missing in p67phox mutant mice [128]. Whether this action of p67phox is specific for the Dahl rat and whether it is mediated by $\bullet O_2^-$ -dependent scavenging of NO has not yet been determined.

To our knowledge the only study addressing the importance of p67phox in the vascular system outside of the kidney was published by the group of P. Pagano. A dominant negative construct of p67phox, which was applied by virus-based overexpression in the adventitia was used to study the contribution of this compartment to redox-mediated vascular pathology [129]. This approach reduced neointimal hyperplasia to some extent in the carotid wire injury model of rats but much less effective than the p47phox-Nox2-interaction inhibitor previously generated by the group [129]. Whether this was a consequence of an insufficient delivery of the dominant-negative construct or a less important role of the adventitia or p67phox in this system is not known.

Collectively, although a lot of mechanistic research has been published on the mode of activation of the leukocyte NADPH oxidase by p67phox, the vascular role of the protein has not sufficiently been studied. No patient data are available for the vascular system and work in transgenic mice has not yet been published.

4.2.4. NoxA1

The situation for NoxA1 is somewhat similar. No patient or knockout data are available up to date. Transfection of an anti-sense plasmid for NoxA1 attenuated the ROS production and agonist-induced gene expression of cultured mouse aortic smooth muscle cells [74] and also the agonist-stimulated ROS production of endothelial cells was reported to be sensitive to an siRNA against NoxA1 [103]. The only in vivo work comes from M. Runge's group. In that study, adenovirus-mediated overexpression of NoxA1 in guidewire-injured mouse carotid arteries significantly increased superoxide production in medial VSMCs and enhanced neointimal hyperplasia [104]. Whether a loss of function of NoxA1, however, has the opposite effect, is not known. It therefore has to be concluded that the contribution of endogenous NoxA1 for vascular ROS production as well as it vascular function are basically

still unknown.

4.2.5. p40phox

p40phox is certainly a special cytosolic Nox protein as its mechanistic contribution to Nox activation is not well understood. Only a single paper has been published on the vascular function of this subunit. This suggests that p40phox and p47phox may substitute for each other in coronary microvascular endothelial cells of mice on a 129sv background. Knockout of p47phox increased the level of p40phox expression, whereas depletion of p40phox in wild-type cells increased p47phox expression [105]. It was suggested that p40phox is phosphorylated in resting endothelial cells and contributes to basal ROS formation [105]. Collectively, more work is needed to establish a vascular function of p40phox.

5. Functions of cytosolic proteins beyond Nox activation?

P47phox and NoxO1 contain SH3 domains, which are frequent interacting domains. Also their dependency on phosphatidyl-inositol-phosphates for membrane translocation through their PX-domain is not a unique feature of these proteins. It could therefore be inferred that these two proteins have functions beyond Nox activation. With respect to publications, however, little has been presented to support this notion. In dendritic cells, p47phox has a ROS-independent negative feedback function in TLR-9 signaling [130]. In smooth muscle cells, p47phox has been shown to interact with cortactin [131] and interestingly, some evidence has been presented that this interaction in the heart promotes a ROS-independent reorganization of the cytoskeleton [132]. Although this particular work is interesting, the true nature of the function of p47phox deletion, its impact on Rac1 and the role of NoxO1 have not been studied. Thus, ROS-independent function of cytosolic Nox proteins are not well established and insufficiently studied at large but also in the cardio-vascular system.

6. Pharmacological exploitation

Based on the concept that ROS are potential harmful molecules, NADPH oxidases have been a pharmacological target since long [133]. The therapeutic strategies take advantage of cytosolic Nox activating proteins. Rac is a target of statins and several inhibitors [134] and p47phox is thought to be inhibited by the natural compound apocynin [135–137]. The compound, however, is notoriously unspecific and numerous papers have been published about numerous Nox independent effects like inhibition of rhoA [138] and antioxidant actions [139].

For mechanistic insights into the mode of interaction of the different subunits, peptides were developed as recently reviewed by Pick's [140]. Pagano developed a Nox2 inhibitory peptide, which blocks the interaction with the cytosolic subunits towards pharmacological applications. By fusing a HIV-tat sequence to the inhibitory peptide gp91ds, gp91ds-tat was obtained, which is taken up by cells and has strong Nox2 inhibitory effects [141]. Gp91ds-tat has shown remarkable effectivity in numerous animal and cell culture studies of the cardio-vascular system. It prevents angioplasty-induced neo-intima formation [141], blocks endothelial dysfunction in reno-vascular hypertension [142], prevents vascular stretch-induced ROS formation and myogenic tone [143] and prevents neurovascular dysfunction during aging [144], just to name a few examples. Due to its unique peptide sequence, gp91ds-tat is considered specific for the Nox2/p47phox NADPH oxidase. Based on this Pagano and his group also developed an inhibitor preventing the interaction of NoxA1 with Nox1 named NoxA1ds [145]. First data suggest that the compound prevents hypoxia-induced cell endothelial cell migration [145] and stretch-induced ROS production in smooth muscle cells [146] but more data are needed to determine whether NoxA1ds is as potent as gp91ds-tat. Recently, another effective peptide directed against Nox1 has been published [147], whereas this compound has effects in the cardio-vascular system, however, is not known.

In the small molecule field, D. Lambeth's group developed ebselen derivatives which appear to specifically block the interaction of p47phox and Nox2 [148]. Ebselen itself reacts with thiol groups and forms mixed seleno-sulfur-bridges [149]. Since Nox2/p47 interaction also relies on reduced thiol groups in the binding region, it might be possible that ebselen derivatives interfere with these thiol groups. Although to our knowledge these substances have not been tested in the cardio-vascular system in vivo, they demonstrate feasibility of an orally active interaction inhibitor and are of much broader utility than the peptide inhibitors. The latter have to be injected, exhibit a low half-life and may raise an immune-response. A major disadvantage of ebselen as a therapeutic agent is that it inhibits many thiol-dependent enzymes, which could also contribute to its liver toxicity.

It should be mentioned that numerous other compounds block Nox-dependent ROS production through an action on the cytosolic subunit. Given that phosphorylation of p47phox is a critical step in activation, particularly inhibitors of protein kinase C and of PI3-kinase prevent Nox activation which has been exploited pharmacologically [1]. As these inhibitors, however, have numerous targets, they should not be considered bona fide Nox specific. An excellent review of NADPH inhibitors has been published in 2012, which covers additional aspects which go beyond the focus of the present article [150].

7. Concluding remarks

NADPH oxidases are pivotal players of redox-regulation and mediators of oxidative stress. In the cardio-vascular system an impressive body of literature has documented a pronounced importance of Nox1 and Nox2. Both enzymes depend to cytosolic activator proteins, but still fairly little is known about these cytosolic proteins. Reliable data on protein expression applying rigorous controls like siRNA and knockout samples are rare and an in depth characterization of the cardiovascular function of NoxA1 and p67phox is quasi non-existent. Despite this, targeting the interaction of the cytosolic proteins with the membrane-bound Nox has emerged as an attractive way to specifically block Nox activation. Before this knowledge, however, can be translated into the clinical setting, the crosstalk between the cytosolic subunits should be better defined and it should be addressed whether these proteins have important additional functions beyond Nox activation.

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Disclosures

The authors declare that they have no conflicts to disclose.

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