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Review Article

Nox family NADPH oxidases: Molecular mechanisms of activation

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ABSTRACT

NADPH oxidases of the Nox family are important enzymatic sources of reactive oxygen species (ROS). Numerous homologue-specific mechanisms control the activity of this enzyme family involving calcium, free fatty acids, protein–protein interactions, intracellular trafficking, and posttranslational modifications such as phosphorylation, acetylation, or sumoylation. After a brief review on the classic pathways of Nox activation, this article will focus on novel mechanisms of homologue-specific activity control and on cell-specific aspects which govern Nox activity. From these findings of the recent years it must be concluded that the activity control of Nox enzymes is much more complex than anticipated. Moreover, depending on the cellular activity state, Nox enzymes are selectively activated or inactivated. The complex upstream signaling aspects of these events make the development of "intelligent" Nox inhibitors plausible, which selectively attenuate disease-related Nox-mediated ROS formation without altering physiological signaling ROS. This approach might be of relevance for Nox-mediated tissue injury in ischemia–reperfusion and inflammation and also for chronic Nox overactivation as present in cancer initiation and cardiovascular disease.

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Contents

Abbreviations: AIR, autoinhibitory region; AMPK, AMP-dependent kinase; BMP, bone morphogenic protein; cAMP, cyclic adenosine monophosphate; CO, carbon monoxide; CyPA, cyclophilin A; DPI, diphenylene iodonium; EGF, epidermal growth factor; GEF, guanine nucleotide exchange factor; HCAEC, human coronary artery endothelial cells; HO-1, heme oxygenase-1; HUVEC, human umbilical vein endothelial cells; I/R, ischemia/reperfusion; LPS, lipopolysaccharide; PAK-1, p21-activated kinase; PDI, protein disulfide isomerase; PI3K, phosphatidyl inosite 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLA2, phospholipase A2; 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

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Introduction

The NADPH oxidases of the Nox family are important sources of cellular reactive oxygen species (ROS). Nox enzymes gained considerable scientific interest and a PubMed research in June 2014 yielded almost 14,000 publications touching the topic. Nox-derived ROS contribute to chemical modification of molecules involved in hormone formation and matrix modification, to host defense, to redox signaling, and probably many other events [1]. If counted by the number of large transmembrane catalytic subunits, 7 Nox homologues are present in the human genome: Nox1 to Nox5 and Duox1 and Duox2. These differ in their expression level, expression control, organ-specific expression, type of ROS release, and in the control of their activity. Obviously, the latter aspect is of particular importance with respect to the acute control of ROS formation and to the association of Nox enzymes with signal transduction. As considerable progress has been made over the past years in dissecting the different mechanisms of activity control of Nox1 to Nox5, this review will focus exclusively on this aspect. More than 3000 publications concern the topic of NADPH oxidase activation and therefore this article can only provide a small overview, covering the general principles of Nox activation and highlighting some aspects which the authors feel are particularly important, paradigmatic, or controversial.

General structure of Nox NADPH oxidases

ROS formation by Nox enzymes takes place at the large transmembrane Nox protein. Of these, 7 different homologues have been identified in the mammalian genomes and also plants and lower organisms express Nox enzymes [2]. Here we will focus on mammalian Nox enzymes which are termed Nox1 to Nox5 and Duox 1 and 2, which is also the name of the large catalytically active subunit.

This transmembrane protein when present as a naïve monomer is inactive and interaction partners are required for its maturation, stabilization, heme incorporation, and correct trafficking to its physiological site of activity. Nox1, Nox2, and Nox3 [3] interact with the small transmembrane protein p22phox, and although Nox4 also shows this interaction [4], it appears to be somewhat different than the other Nox enzymes [5]. Nox5 does not interact with p22phox but rather appears to homo- or multimerize [6]. Although p22phox has a scaffold function for the maturation and the folding of the catalytically active Nox protein through a proline-rich region it also provides a binding platform for the cytosolic activator proteins of Nox1 to Nox3. In line with the exclusive dependency of Nox1 to Nox3 on cytosolic activators, mutations in the proline-rich region of p22phox do not affect Nox4- and Nox5-dependent ROS production [7]. Duox1 and 2 require Duoxa1 and Duoxa2 as a scaffold for their maturation and proper function [8]. In addition to these structural components of the enzyme, at least Nox1, Nox2, and Nox3 require interaction with cytosolic proteins for activation.

General mechanisms of activation

The classic activity control of Nox enzymes is exerted by calcium or protein–protein interactions and concerns all Nox enzymes, with the exception of Nox4, which is constitutively active [9–11]. Nox5 and the Duox enzymes contain EF hands making them directly calcium dependent [12,13]. In contrast, Nox1 to Nox3 are not directly calcium sensitive, although calcium is involved in the upstream mechanism of their activation. Two main elements contribute to this process: interaction with the small GTPase Rac and interaction with cytosolic activator proteins. So far the Nox activators Noxa1 and p67phox have been identified to require organizing scaffolding proteins to tether them to the large Nox enzyme—these are termed p47phox and Noxo1 [1]. In essence, the activity control of Nox1, Nox2, and Nox3 is therefore primarily achieved through alterations of the cytosolic proteins. In addition to these established mechanisms, recently novel mechanisms of activity control have been identified as will be discussed below. Whether the activity of Nox4 is subject to any control is still unclear (Fig. 1).

Leukocyte NADPH oxidase Nox2—The paradigm for NADPH oxidase activation

Under resting conditions, the phagocyte NADPH oxidase is inactive. Stimulation of formylated peptide receptors or of Fc receptors activates the oxidase which leads to an O_2^- production

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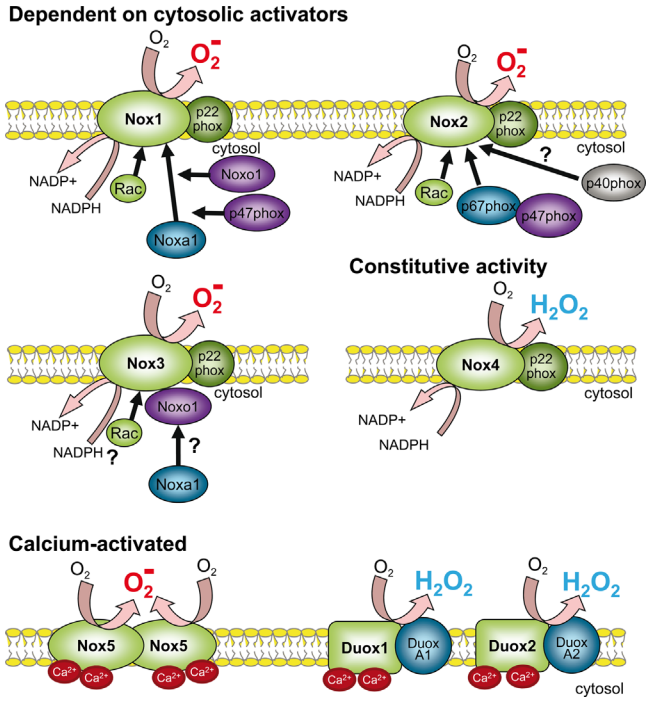

Fig. 1. Composition of Nox enzyme complexes.

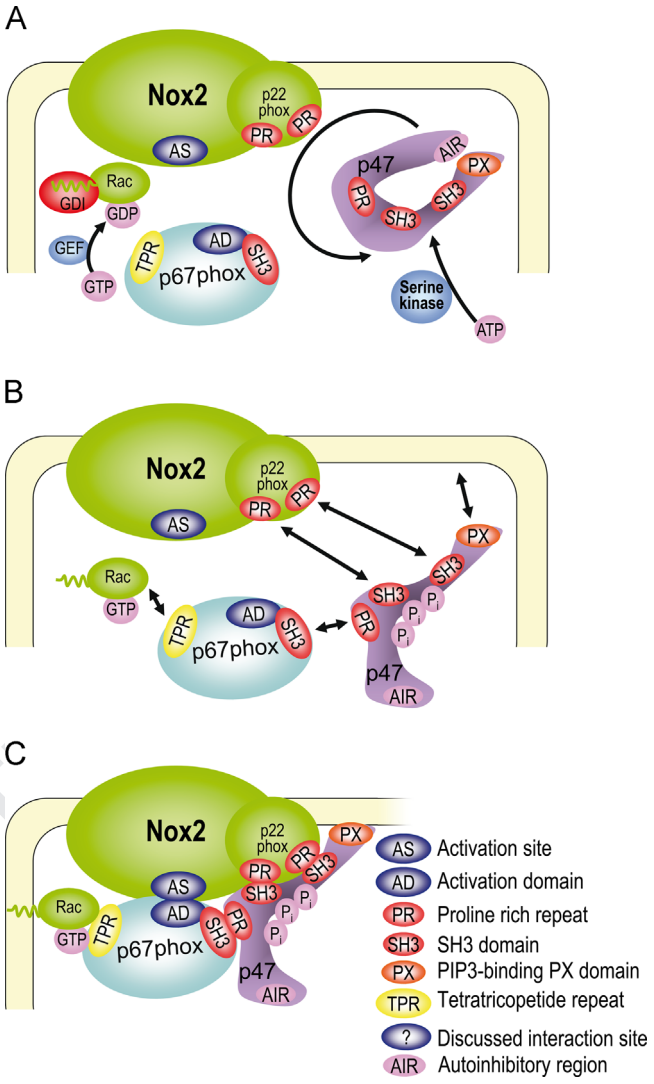

Fig. 2. Activation of Nox2.

of approx. 10 nmol/min/10^6 cells. Despite some remaining controversies, it is certain that the cytoplasmic subunit p67phox must interact with Nox2 for this [14,15] and that active Rac is required [16].

The activation of Nox2 by p67phox occurs via a domain located in the region from amino acids 200–210 close to the N-terminal part of p67phox [17]. The interaction at this site, however, is not strong enough to allow steady association of the two molecules. It is therefore supported by p47phox, which acts as adaptor protein. In essence, active p47phox binds p22phox [18,19] and thereby approximates p67phox to Nox2.

This function of p47phox is governed by serine phosphorylation, which occurs during activation of the oxidase [18]. Nonphosphorylated p47phox does not interact with the membrane and other oxidase subunits as the bindings of its bis-SH3 domain and the PX domain to their targets are functionally inhibited by an autoinhibitory region (AIR) [20]. Serine phosphorylations at positions 303, 304, and 328 result in a conformational change of p47phox and in the exposure of the interacting domains [20]. Subsequently, the bis-SH3 domain of p47phox binds to the proline-rich region (PRR) of p22phox [20,21], whereas the PX domain allows anchoring of p47phox in the membrane [22]. P67phox interacts via its C-terminal SH3 domain with the C-terminal proline-rich region of p47phox [23] at an unusual high affinity of 20 nmol/L [24].

The small GTPase Rac1 is also critical for oxidase activation. Rac-GTP interacts with the oxidase via the TPR motif in the N-terminal part of p67phox [25]. Membrane binding of Rac does not require p47phox, as active Rac anchors in the membrane via its prenylated tail. Indeed, high amounts of Rac have been shown to cause translocation of p67phox to the membrane even in the absence of p47phox [26]. In vivo, the translocation of Rac is independent of that of p47phox and p67phox and still takes place in patients lacking NADPH oxidase expression [27] (Fig. 2).

Nox1, the colonic NADPH oxidase system

Nox1 is particularly highly expressed in the epithelial cells of the gastrointestinal tract [28]. The search for cytosolic Nox1 interacting proteins revealed that Nox1 is preferentially activated by a protein combination consisting of Noxa1 and Noxo1 [29–31]. Like Nox2, Nox1 forms complexes with p22phox, and p22phox is required for Nox1-dependent O_2^- formation [31]. Transfection of Nox1 together with the p47phox homologue Noxo1 and the p67phox homologue Noxa1 results in constitutive O_2^- production. This basal activity is a consequence of the lack of the AIR in Noxo1 [32], which results in constitutive binding of Noxo1 through its PX domain to PtdIns(4,5,)P2 and PtdIns(3,4,5,)P3 lipids in the plasma membrane [33]. Removal of the PX domain prevents membrane interaction of Noxo1 [34]. Noxo1 therefore provides a scaffold that binds both Noxa1 and p22phox, thereby increasing the efficiency by which Noxa1 interacts with Nox1. The latter interaction is a prerequisite for Nox1 activation. Disruption of the binding of Noxa1 to Nox1 by the inhibitory peptide NoxA1ds, already at nanomolar concentrations, blocks Nox1 activation [35].

Despite this constitutive activity, some control of Nox1-dependent ROS formation occurs. Like Nox2, the activity of Nox1 is Rac dependent, but increases Nox1 activity to a lesser extent than that of Nox2 [36–38]. Similar to p47phox, Noxo1 interacts with both of its SH3 domains with p22phox and thus forms a super-SH3 domain but the interaction for Noxo1 is considerably weaker, suggesting autoinhibitory mechanisms. Also the interaction of Noxo1 with Noxa1 is weaker than that of p47phox with p67phox [39]. Most interestingly, however, the interaction of

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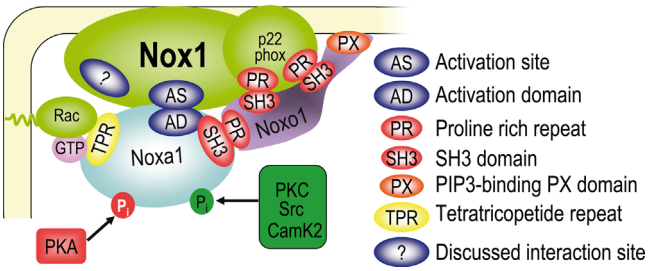

Fig. 3. The active complex of Nox1. P_i in green indicates activating phosphorylations, whereas red denotes inhibition. PKA, protein kinase A; PKC, protein kinase C; CamK2, calcium-calmodulin-dependent kinase 2.

Noxo1 with p22phox in humans is much weaker than that in the mouse proteins which could be interpreted as a sign for an autoinhibitory element present in the human Nox1 complex [39]. In line with this, activation of the protein kinase C by phorbol ester increases ROS formation by the human but not the murine Nox1 complex [29–31] and it is attractive to speculate that this is a consequence of a phosphorylation event in the human Nox1 complex, which strengthens the binding of the membrane subunits to the cytosolic activator proteins (Fig. 3).

Mixed Matters

In addition to the prototypic complexes Nox2–p47phox–p67phox and Nox1–Noxa1–Noxo1, obviously also other combinations of the three proteins are possible. Oxidase activation in the alternative combinations occurs but is considerably weaker. Nox1 expression in Nox2-deficient leukocytes in part restores their p47phox/p67phox-dependent ROS formation [40] and Nox2 can also be activated by the combination of Noxa1 and Noxo1. In reconstitution experiments the V_{max} was only 30% of that achieved using p47phox and p67phox, and higher concentrations of Noxo1 and Noxa1 were needed compared to activation with p47phox and p67phox [41]. One reason for the reduced activity might be that a residual autoinhibitory capacity is present in Noxa1. C-terminal truncation increased the ability of Noxa1 to activate Nox2 without changing the binding to Rac and Noxo1 in a reconstitution system [42]. Moreover, different splice variants of Noxo1 have been reported which differ in their ability to activate Nox2 but not Nox3. These also differ in the capacity to interact with plasma membrane phosphoinosite lipids [43].

The biological occurrence and relevance of the alternative complexes is not well studied. In murine smooth muscle cells, p47phox is expressed but p67phox is missing and it appears that a complex of Noxa1 and p47phox serves to activate Nox1 [44,45]. Importantly, the AIR of p47phox also prevents its binding to Noxa1 which renders this type of ROS formation agonist stimulated [46].

Interestingly, in vascular smooth muscle cells of p47phox-/- mice, the basal ROS formation is increased, whereas the AngII-induced stimulated ROS production is missing. It is tempting to speculate that subunits which, due to the presence of p47phox, can usually not approach the oxidase, mediate this effect [47]. These might be residual amounts of Noxo1 or p40phox. In leukocytes, p40phox is highly abundant and required for efficient receptor-dependent stimulation [48]. In microvascular endothelial cells, it has been suggested that p40phox contributes to basal activity and thus ROS production of Nox2 [49]. In neutrophils, p40phox primarily facilitates FcγR-induced Nox activation but not complex assembly. It stimulates ROS production via a PIP3 signaling after phagosome internalization [48]. Similar to p47phox, p40phox carries a PX domain, which localizes the protein to early endosomes if overexpressed and fused to GFP. p40phox, similar to p47phox, binds and translocates p67phox [50]. In the cell-free assay of leukocyte membranes phosphorylated p40phox acts as negative regulator of NADPH oxidase if p47phox is present [51], whereas in the absence of p47phox it is able to also activate Nox2 [52].

Specific aspects of activation

Phosphorylation

Phosphorylation of p47phox

As noted above, an essential step for the activation of the classic Nox2-containing NADPH oxidase is serine phosphorylation of p47phox on residues 303, 304, and 328. Different protein kinase C (PKC) isoforms have been suggested to be involved in this process, depending on the cell type and agonist studied. For neutrophils and monocytes a central role of PKCδ has been suggested [53], and also PKC α, β II, and ζ have been shown to phosphorylate p47phox at multiple serines [54]. In neutrophils stimulated with fMLP, p21-activated kinase (PAK-1) initiates activating p47phox phosphorylations [55] but the overall function of PAK-1 is unclear. In myocytes, for example, the enzyme appears to limit rather than enhance ROS formation [56]. Finally, also protein kinase B/AKT has been suggested to activate p47phox through phosphorylations in leukocytes [57,58] and endothelial cells [59] (Fig. 4).

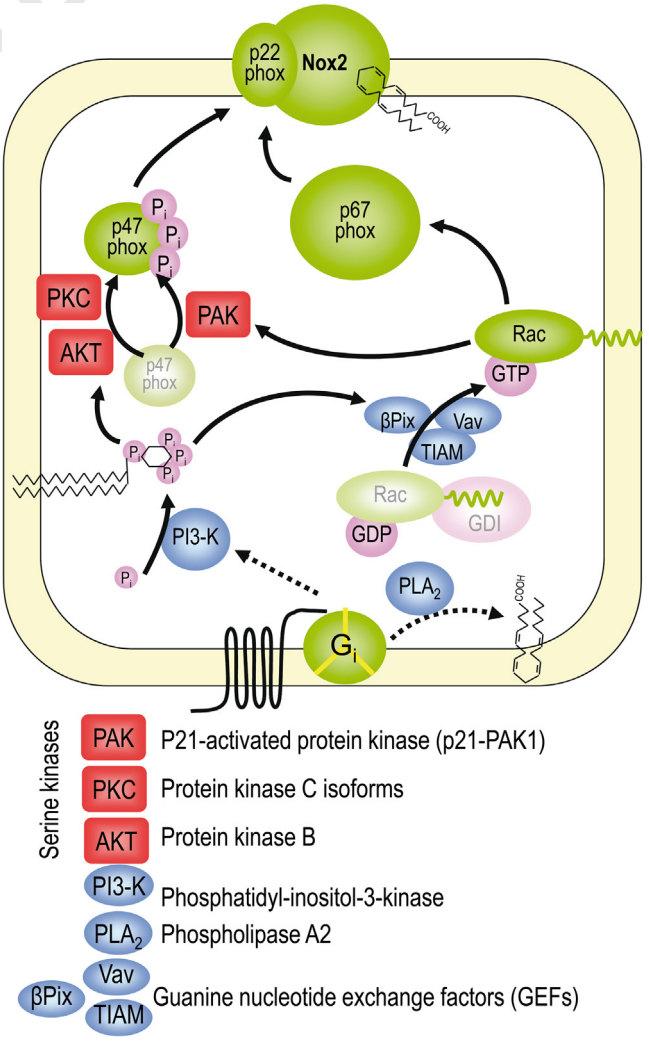

Fig. 4. Activation of Nox2 in leukocytes.

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In addition to activation, other phosphorylation events control the speed, duration and strength of the activation event and thereby prime the Nox2 containing oxidase for maximal activity. Finally, some additional phosphorylation events have been reported to increase overall activity. In contrast to activation, priming is mediated by the MAP kinases ERK1/2 and p38 and concerns Ser345 of p47phox [60–62]. Priming occurs in response to cytokines such as IL-18 or in neutrophils TNFα [62], which themselves are not able to activate the oxidase.

Phosphorylations beyond p47phox

Almost every Nox subunit has now been shown to be subject to functional relevant phosphorylation. For p22phox, a phosphorylation by phosphatidic acid-activated protein kinase was reported [63] and also PKCs can phosphorylate p22phox at Thr147, a process which promotes the interaction with p47phox [64]. Also, Nox2 is phosphorylated in response to PKC stimulation to increase maximal ROS production [65]. In neutrophils, p67phox is constitutively phosphorylated by a serine/threonine kinase, but the site and the relevance of this has not yet been determined [66]. Additionally, a phosphorylation by PKCδ has been reported in monocytes, which may limit enzyme activation. It has not been clarified yet whether this phosphorylation is monocyte-specific nor has the site of phosphorylation been identified [67].

Potentially as a consequence of the lack of the AIR in Noxa1, significant work has been devoted to the identification of a potential phosphorylation-dependent activity control of Noxo1 and Noxa1. cSrc phosphorylates Noxa1 on Tyr110 to increase ROS formation in a colon cancer cell line [68]. cSrc also phosphorylates Tks proteins and Tks4 and Tks5 directly interact with Noxa1 through the PRR to promote its activation, whereas they do not interact with p67phox [69]. Noxa1 is also phosphorylated at Ser154 and this is required for optimal activity. Whether the phosphorylation at this site is constitutive or modulated in response to stimuli is controversial [70,71]. For Noxa1, inhibitory phosphorylations have also been reported: Protein kinase A phosphorylates Noxa1 at Ser172 and Ser46, which results in increased 14-3-3 zeta protein binding and inhibition of the enzyme in HEK293 cells [72] and a similar activity was reported for PKC and NoxaSer172 [73]. PKA belongs to the system endogenously limiting Nox-dependent ROS production; this aspect is further detailed below. Additional inhibitory phosphorylations of Noxa1 occur in response to growth factor-dependent MAP kinase stimulation on Ser282 [73,74]. Also Cam-kinase 2 (CamK2) appears to be involved in NADPH oxidase activity control by phosphorylation. Although the targets of CamK2 have not been identified, yet, inhibition of the enzyme attenuates cerebral ischemia-induced ROS formation and tissue damage [75]. In CHO cells, phorbol ester induces phosphorylation of Noxo1 at multiples sites. Noxo1 Thr341 is a direct PKC target ex vivo and a phosphomimetic alanine substitution increased ROS production and interaction with Noxa1 [70] (Fig. 3).

Activation by lipids

Through the stimulation of upstream activating cascades, lipids activate NADPH oxidases. In addition, several older reports suggest that arachidonic acid and potentially phosphatidic acid are potentially directly involved in Nox activation [6,76]. The molecular mechanism of activation for both lipids is, however, controversial as the amphiphilic nature of the molecule might mimic the activating detergent effect observed for p47phox in the cell-free Nox activity assay [77,78], and similar reports have been published for Noxo1 [46]. For phosphatidic acid it was thought that the lipid activates the phosphatidic activated protein kinase which then phosphorylates p22phox [63]. As this enzyme is calcium dependent and as the effects of phosphatidic acid are still observed under calcium-free conditions, it was suggested that the lipid may also directly act on the Nox enzyme activity [6]. In the case of arachidonic acid, the observation that only the *cis* form activates Nox2, whereas the *trans* form of this lipid is rather inhibitory, suggests that the action of arachidonic acid extends beyond a simple detergent-mediated displacement of the AIR in p47phox [79]. Arachidonic acid is provided through the action of phospholipase A2 (PLA2), which releases this polyunsaturated fatty acid from membrane stores and a cytosolic phospholipase A2α interacts with the cytosolic Nox proteins in neutrophils [80]. Some observations indicate that this enzyme might be peroxiredoxin 6, an atypical peroxiredoxin which harbors also PLA2 activity. The enzyme is required for maximal turnover and ROS production in the membrane assay of leukocytes [81,82] and for agonist-stimulated ROS production of endothelial cells [83]. Arachidonic acid delivery to the Nox enzyme requires calcium-binding S100 proteins [84]. Interestingly, in neutrophils missing Rac2, exogenous application of arachidonic acid leads to oxidase activation [85], suggesting that the lipid can in part replace the action of Rac on p67phox. As a potential mechanism interaction of arachidonic acid with the C-terminal SH3 domain of p67phox has been discussed. It is thought that this part of the protein has some endogenous negative regulatory function which must be attenuated by phosphorylation or amphiphiles [86]. Recently, nitro-lipids have gained considerable attention as activators of the antioxidant response element transcription factor Nrf2. Nitroarachidonic acid may, however, also have a direct antioxidant effect on Nox enzymes as it prevents oxidase assembly and superoxide formation in macrophages independent of p47phox phosphorylation [87]. It is, however, unclear whether this arachidonic acid derivative displaces the naïve lipid from its binding site.

Interaction of p47phox with the cytoskeleton

Actin enhances ROS formation by NADPH oxidases in the cell-free system. p47phox indeed contains actin binding sites and interacts with cortical actin in the cell-free system [88]. Disruption of the actin cytoskeleton with cytochalasin B attenuates AngII-induced ROS formation, MAP kinase interaction, and p47phox translocation in VSMC [89]. It should, however, be noted that in human monocytes, cytochalasin D even increases Nox2 activity and p47phox membrane translocation [90]. In vascular smooth muscle cells (VSMC), p47phox interacts with cortactin [91]. This is required for AngII-induced ROS formation and also mediates mechanical stress in heart failure [92]. A role of the prooxidative cyclophilin A (CyPA) has also been suggested in the context: The protein possesses peptidyl-prolyl *cis–trans* isomerase activity and scaffold function and is required for AngII-induced ROS formation. Through its isomerase activity CyPA appears to facilitate the interaction of p47phox with the cytoskeleton [93]. Finally, the cytoskeleton through a PKCδ-dependent mechanism controls p47phox phosphorylation in VSMC: After AngII stimulation, smooth muscle 22α (SM22α) is phosphorylated, which then releases PKCδ. This subsequently phosphorylates p47phox resulting in Nox activation [94].

General modulators of activity

The overall activity of a Nox enzyme not only depends on a successful activation but also on some more general factors controlling enzyme activity. A central aspect is a sufficient supply of the substrates NADPH and oxygen and indeed in phagocytes 6-phosphogluconate dehydrogenase directly interacts with Nox2 to fuel the enzyme with NADPH [95]. NADPH supply can be a limiting

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factor for oxidase activity in the setting of glucose-6-phosphate dehydrogenase deficiency in the vascular system [96,97] and also for hepatic Nox4 activity in the nucleus [98]. Similarly, oxygen is also a limiting factor. The Nox2-dependent system is adapted for inflammatory regions with a low pO_2 (K_m for oxygen 36 μmol/L approx. FO_2 3.1%) [99], whereas the K_m for Nox4 for oxygen is 220 μmol/L (approx. FO_2 18%) [100], making this enzyme exquisitely oxygen sensitive. This finding supports a previous observation of a direct relation of oxygen tension and Nox4-dependent H_2O_2 production in muscle [101], which subsequently leads to an oxygen-adapted oxidation of cysteines within the ryanodine receptor [102].

Maturation of Nox enzymes is required for its activity, correct folding and trafficking. Although Nox1 and Nox2 are glycosylated during maturation, this does not impact on the function of the enzyme nor its activation [103,104]. Heat shock protein 90 (HSP90) appears to be involved in one of these processes as it binds to the C-terminal parts of Nox1, Nox2, Nox3, and Nox5 and facilitates $O_2^{\cdot-}$ formation [96]. As Nox4-dependent H_2O_2 production is HSP90 independent, this might relate to possible activation mechanisms of the agonist-stimulated NADPH oxidases. Loss of Hsp90 leads to degradation through Hsp70 and the Hsp70-regulated ubiquitin ligated "carboxyl terminus of Hsp70 interacting protein" (CHIP) [105].

Another family of chaperones, the protein disulfide isomerases (PDI), at least also support Nox activity. PDI coprecipitates and colocalizes with Nox enzymes and downregulation of PDI attenuates Nox-induced ROS production in VSMC [106], endothelial cells [107], and leukocytes [108]. PDI interacts not only with the catalytically Nox subunit but also with p47phox [106,108], potentially indicating that its thioredoxin function keeps these redox-sensitive subunits in an active state. P47phox contains four cysteines and replacing them with alanines [109] or alkylation by *N*-ethylmaleimide (NEM) [110] blocks p47phox activity and trafficking. Moreover it was previously observed that the cysteine-oxidizing phenyl-arisine oxide reduces Nox activity of leukocytes [111]. Similarly, thiol oxidation by diamine attenuated smooth muscle Nox activity [112]. Nox4 also is inactivated by alkylation [113]. Finally, p67phox is also oxidation sensitive: Irradiation of the purified subunits induces inactivation of the NADPH oxidases and the most sensitive part is potentially the C-terminal SH3 domain of p67phox as determined by radiation experiments [114]. This collection of findings illustrates the paradox situation that despite the production of ROS, NADPH oxidases themselves are ROS sensitive. The enzymes are, however, simultaneously equipped with the machinery to keep them in an active reduced state, for example, by PDI-mediated thiol reduction. Finally, it should be noted that also H_2O_2 actives NADPH oxidases [115,116]. In PMNs, this occurs through a pathway involving calcium, Abl-kinase, and Thr311 phosphorylation of PKC δ [117], but different mechanisms are likely to be operative in other cells.

Activity control by GTPases

GTPases contribute to Nox activation in a complex manner. Rac is a critical component in the activation of Nox1 and Nox2. For Nox3 the situation is less clear and although a Rac binding site was reported in Nox3 [37], functional studies in overexpression systems failed to detect Rac-dependent Nox3 activation [118]. Nox4 does not contain Rac binding sites and is not stimulated by Rac in Cos-Phox cells [5]. Of the two main homologues present, Rac2 is restricted to PMNs, whereas Rac1 is ubiquitously expressed. The activity control of Racs is central for Nox1 and Nox2 activity and is involved in almost all signals increasing Nox activation, such as growth factors, cytokines, and also chemophysical stimuli such as shear stress [119]. If Rac1 is inhibited pharmacologically by statins or toxins, no Nox2 activation occurs in response to phorbol ester stimulation [120] and statin withdrawal results in an overshoot of Nox stimulation, resulting in inflammation and oxidative stress [121,122].

At least in cell-free systems Rac2 and Rac1 are interchangeable in activating Nox2 and both can also bind Noxa1 to activate Nox1 [119]. Rac binding sites have been identified in Nox1, Nox2, and Nox3 but not Nox4 [123]. Indeed, in leukocytes Rac2 cooperates with p47phox to bind p67phox to Nox2 [119]. A similar mechanism is operative for Rac1 and Nox1: The GTPase directly interacts with Noxa1 and simultaneously binds Nox1 [124]. Through this mechanism, Rac1 supports membrane localization of Noxa1, as demonstrated by Noxa1 mutants, which does not bind Noxo1 [38].

Despite the apparent promiscuity of the system, Rac2 binds p67phox 6-fold times more strongly than Rac1 and also induces stronger Nox2 activation in neutrophils. In monocytes, Rac1 in contrast is the most important oxidase activator [125]. Interestingly, in Rac2-/- neutrophil progenitors, reintroduction of Rac2 but not Rac1 restores fMLP-induced $O_2^{\cdot-}$ formation and chemotaxis. In contrast, deletion of Rac1 in leukocyte impairs actin assembly and chemotaxis without affecting ROS production in response to PMA and fMLP [126]. The other Rac isoforms, Rac1b and Rac3, have not been well characterized with respect to their function in Nox2 activation. Thus, Rac2 mediates agonist-stimulated ROS production in neutrophils, whereas in other cells, Rac1 facilitates this. It is still not well understood why Nox1-dependent ROS production is frequently observed to be Rac dependent whereas in overexpression studies, the transfection of Nox1, Noxo1, and Noxa1 is sufficient to induce ROS formation. Potentially, high abundance of the cytosolic activating factors renders Rac1 dispensable [127]. Accordingly, Noxa1 can support Nox1 activation in the absence of Noxo1 when targeted to the membrane by a Rac1 membrane anchor [37].

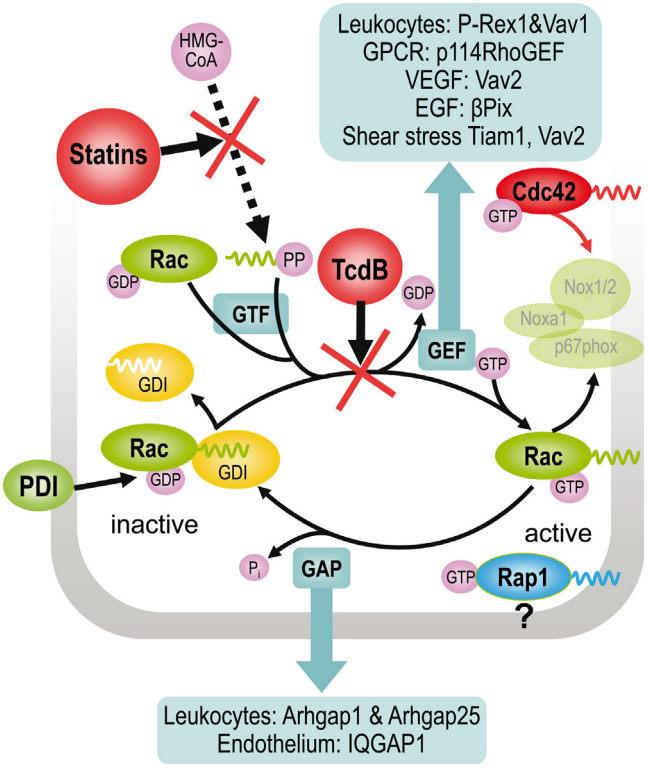

Fig. 5. Activity control of Rac. GEF, guanine nucleotide exchange factor. GAP, GTPase activating protein; GDI, guanine nucleotide dissociation inhibitor; GTF, guanine farnesyltransferase; PDI, protein disulfide isomerase; TcdB, *Clostridium difficile* lethal toxin B.

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Cdc42 is a small GTPase with some functional similarities to Rac with respect to cytoskeleton control. In Nox enzymes, Cdc42 acts as negative regulator as it competes with Rac1 and Rac2 for Nox binding via its insert region [128]. Other GTPases interact with Nox enzymes in an indirect way. Rab GTPases control endosomal trafficking, sorting, and membrane localization and also the GTPase Rap1 was copurified with Nox components [129]. The specific role of this enzyme is still under investigation and it is unclear at what position Rap1 intercepts with the upstream activation pathways of Nox enzymes. Genetic deletion of Rab1a reduced the fMLP-stimulated activation of leukocytes and their ROS production [130] and downregulation of Rap1 reduced Rac activation in macrophages in response to zymosan [131]. In contrast to this, Rap1 is a negative regulator of NADPH oxidase activity in retina pigment epithelial cells [132]. Certainly, more work is needed to understand the complex upstream functions of this interesting GTPase.

Activity control of Rac

Rac belongs to the family of Rho GTPases and is therefore activated by guanine nucleotide exchange factors (GEFs). Rac inactivation occurs through GTPase activating proteins (GAPs) and RhoGDI maintains Rac in an inactive state in the cytosol. [133]. Most research in the field focused on GEFs, although for leukocytes it was reported that Arhgap1 and Arhgap25 are involved in Rac2 inactivation [134]. GEFs exhibit cell-specific expression levels and a stimulus-specific activation pattern through their interaction with G-proteins as in the case of p114RhoGEF or with PIP3 as in the case of P-Rex1. Experiments in Cos-Phox cells revealed that the GEFs Vav1, Vav2, and Tiam1 can stimulate Nox-dependent ROS production [135], whereas a similar activity of P-Rex [136] and β-Pix [36] was established through other means.

In leukocytes, particularly PI3Kγ [137] and subsequent P-Rex1 [136], activation as well as Vav1 are important. In endothelial cells, Tiam1 [138] and Vav2 [138] mediate Rac/Nox activation in response to shear stress and Vav2 also mediates VEGF-induced Rac1 activation [139]. β-Pix is stimulated by epidermal growth factor or PMA and facilitates Nox1 activation in Caco2 [140] and HEK293 cells [36]. P114RhoGEF activates RhoA and Rac1 through the βγ subunit of G-protein-coupled receptors (GPRCs) and stimulates ROS production in NIH3T3 fibroblasts in response to lysophosphatidic acid [141].

Obviously, given the large number of GEFs and the multiple stimuli that activate an individual GEF, the literature cited here can only cover a small portion and this most complex aspect of Nox activity control requires further research (Fig. 5).

Specific considerations for Nox3 to Nox5

Nox3

Nox3 expression is limited to the inner ear where the protein is required for the formation of otoliths [1,142]. Due to this very focal expression, functional data on Nox3 have been only gathered in overexpression systems. Under these conditions, Nox3 is located in the plasma membrane and its maturation requires p22phox [143]. Nox3 has some basal activity, even in the absence of the cytosolic subunits if overexpressed in CHO, HEK293, or COS-7 cells, but activity is totally dependent on p22phox. Rac1 does not affect the activity of Nox3 but the cytosolic factors can further enhance the activity of Nox3 [118]. Some findings suggest that Noxo1 in the absence of Noxa1 or p67phox is able to activate human Nox3 [127] and in this respect the gamma form of Noxo1 has a lower activity than the beta form [144].

Nox4

The activity of Nox4 has generally been thought to be constitutive and thus mRNA formation determines ROS production [145], although mRNA stability, translation efficiency, and protein stability of Nox4 are also controlled [146]. Structurally, Nox4 forms stable complexes with p22phox [4], although the interaction differs from that of Nox2 and Nox1 [5]. In Cos-Phox cells, the activity of Nox4 is Rac independent [5] and coexpression of Nox4 with p47phox and p67phox or Noxa1/Noxo1 does not increase ROS generation [4,147,148]. In line with this, peptide-based inhibitor approaches interfering with the binding sites to Rac1 or the cytosolic phox proteins had no effect on Nox4 activity [149]. Mutation experiments revealed that the constitutive activity of Nox4 is particularly located in the B-loop and the C-terminal dehydrogenase domain [11]. Analyses of the isolated dehydrogenase domain demonstrated constitutive activity of this part of Nox4 but not of a similar construct of Nox2 and the step from NADPH to FAD appeared to be rate limiting [150]. The K_m for Nox4 for NADPH was calculated at 55 ± 10 μmol/L, which is similar to that of Nox2 (around 50 μmol/L). In these experiments, FAD had to be added and saturation of the fragment was achieved at 25 μmol/L [151]. Interestingly, limiting heme supply to Nox4 may affect the cellular localization of the protein similarly as heme is required for maturation of Nox2 but additional studies are needed to better qualify this aspect [152]. Despite p22phox, these cofactors, and substrates, very few Nox4-interacting molecules have been reported. The protein polydip2 modulates Nox4 activity but no data suggest that this interaction is subject to acute regulations [153]. Another possible interesting interaction facilitates Nox4 degradation. This occurs at least in some part through ubiquitination in a process involving the ubiquitin ligase CBl-c; Hic-5 and HSP27 [154].

All these findings could lead to the interpretation that Nox4 activity is exclusively controlled by the enzyme expression, which rapidly increases in response to hypoxia or transforming growth factor β1 through transcriptional control. Several studies focussing on acute agonist-stimulated ROS production indeed reported that Nox4 only mediates basal cellular radical formation whereas agonist-dependent responses to AngII [155] and bFGF [156], for example, are mediated by other Nox enzymes. Moreover, genetic deletion of Nox4 reduces the constitutive basal ROS formation of murine vessels [157] and kidneys [158].

Nox4: Only constitutively active or also activated?

A growing number of studies suggest that Nox4 might also be acutely activated in response to agonist stimulation, but a detailed analysis of these papers yields a very mixed picture. Often changes in ROS production were attributed to Nox4 on the basis of the much lower mRNA expression of the other Nox enzymes. This view is certainly invalid as careful studies in Nox1 knockout mice clearly demonstrated how important this enzyme can be despite the almost undetectable amounts of Nox1 mRNA present in cells [159]. Moreover, it is ignored that the basal production of H_2O_2 or the siRNA approach, used to down-regulate Nox4, can sometimes affect the expression of the cytokine-inducible Nox1, Nox5, and Duox enzymes. Moreover, the duration of stimulation is often hours rather than minutes so that induction of Nox4 could contribute to the increased ROS formation. Vice versa, altering Nox4-degradation pathways can quickly increase the Nox4-dependent ROS production, but again, this would be due to an increase in the protein amount and not an activation of the

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enzyme [154]. For example, in kidney cells, BMP2 stimulation within minutes transiently increased ROS formation which is associated with a transient rapid increase in Nox4 protein abundance [160].

Some reports attribute an acute Rac-dependent ROS production to Nox4 [161–165], although this is incompatible with the absence of a Rac-binding site in the protein [123,147]. A potential explanation for these findings is that Rac does not directly activate Nox4 but alters pathways which control Nox4 degradation or localization through changes in the cytoskeleton. For example, in rat VSMCs, IGF-1 rapidly increased Nox4 protein expression without affecting mRNA level [166]. Also Rac1 was involved in the increased ROS formation in response to IGF-1. Importantly, however, the authors did not find any evidence for a direct role of Rac1 in Nox4 activity but rather suggest that two different signaling pathways may act in parallel [166]. As Rac1 regulates the cytoskeleton, a potential and interesting explanation is an alteration in the cytoskeleton which changes the NADPH supply to Nox4 or the intracellular localization of Nox4. Also, it is highly possible that Nox4 is subject to posttranslational modifications such as oxidation, sumoylation, acetylation, and phosphorylation. Indeed, as noted above ubiquitination of Nox4 and subsequent degradation were already reported [154]. Translocation of Nox4 might be associated with activation of the enzyme, although these data, due to the limited availability of high quality antibodies, must be interpreted with caution. In 3T3L1 adipocytes, insulin acutely stimulates ROS production by a pathway sensitive to dominant-negative Nox4 and siNox4 [167]. In podocytes insulin stimulates a rapid surface localization of Nox4 [168] and also onset of laminar shear stress mediates an acute Nox4-dependent ROS production [169]. The intracellular localization of Nox4 is the subject of a long-lasting controversy with endoplasmic reticulum and mitochondria as well as nucleus reported localizations. Nevertheless, in a few cell lines Nox4 was detected in the plasma membrane [5,11] and interestingly, providing additional heme to HEK293 cells promoted protein maturation and resulted in an increase in the (low) plasma membrane abundance of Nox4 [152].

In HEK293 cells overexpressing Nox4 a direct interaction of Nox4 and TLR4 has been reported as well as an acute increase in ROS formation by Nox4 in this model [170]. Endogenous Nox2, however, was not depleted and these data have not been confirmed in cells endogenously expressing Nox4 or by other groups. Nevertheless, for human endothelial cells, the groups suggested that the same mechanism is operative [171]. In monocytes an interaction of TLR2 and Nox2 was reported by the same group to be involved in mycobacterial killing [172]. In our hands, global deletion of Nox4 had no impact on the LPS-stimulated LPS-mediated production of cytokines in vivo (K. Schröder, unpublished observations).

As noted above, lipids have some special, yet incompletely understood, function for Nox activity. It was also suggested that arachidonic acid increases Nox4 activity in mesangial cells in response to angiotensin II [161] and that arachidonic acid directly increases Nox4 activity in cardiac fibroblasts [173]. Very recently, we reported that TGFβ induces an acute oxidation and internalization of ENaC in pulmonary tissue. In A549 cells, this response was mediated by a TGFβ-dependent production and phosphatidic acid by phospholipase D and the subsequent phosphorylation of this lipid by PIP5kinase1α. Interestingly, the resulting PtdIns(2,5)P2 contributed to acute ROS formation, which was not observed after siRNA or genetic knockdown of Nox4 [174]. Collectively, these findings in large part support the view that at least some activity control of Nox4 occurs; however, the underlying mechanism is yet to be established.

Nox5

The physiological functions of Nox5 are just emerging [175], but significant mechanistic studies on its activation have been carried out. Nox5 is a special NADPH oxidase to the extent that it forms functional oligomers and is therefore independent of any maturation factors [176]. As no additional factors must be transfected, Nox5 is therefore also easier to study in overexpression systems. Due to the presence of 2 EF hands, the first stimulus identified to increase Nox5 activity was calcium. Although the first EF hand has a noncanonical structure, its calcium binding largely enhanced the binding affinity to the second EF hand. Thus, cooperation is present as revealed by isothermal titration calorimetry. Magnesium ions, in contrast, do not bind Nox5 [177]. The activity of this oxidase is, however, also controlled by phosphorylation as also MAP kinases or protein kinase C by phosphorylating Thr494 and Ser498 induce a strong increase in activity [178,179]. Of the individual PKCs, PKCα and PKCε were both identified to mediate the PMA-induced stimulation of Nox5. PKCθ was reported as being unimportant in this context whereas PKCδ even appeared to act as inhibitor of Nox5. PKCα directly binds Nox5 and the PKC inhibitors GO6976 as well as Ro-320-432 blocked Nox5-dependent ROS production in HEK and COS-7 cells [180]. Overexpressed Nox5 is also phosphorylated by calcium/calmodulin-dependent kinase II on several residues. Of these, however, only the phosphorylation on Nox5β-Ser476 (i.e., Nox5γ-Ser521) has proven to stimulate activity [181]. Finally, phosphatidylinositol(e,5)-bisphosphate modulates Nox5 via an N-terminal polybasic region [182]. Endogenously, Nox5 is expressed in sperm and here the nonreceptor tyrosine kinase c-ABL has been implicated in Nox5 activation in response to H_2O_2 [183]. Thus, Nox5 activity is intensively modulated and not only calcium but also a number of phosphorylation sites control ROS formation by this enzyme.

Specific activation situations

As a consequence of the heterogeneity in NADPH oxidase expression, the radical generation in response to identical stimuli varies between cell types and it often remains unclear which of the Nox homologues become activated and whether the mechanism of activation differs between the Nox homologues.

Inflammatory signaling of toll-like receptors and TNFα

Inflammation is a prototypic situation of oxidative stress and this large topic can only be covered incompletely here. In PMNs, however, the respiratory burst is mainly activated by the tyrosine kinase-coupled FCγ receptor and thus through opsonized particles as well as through ligands of the Gαi-coupled fMLP receptor. Other stimuli result only in a low oxidase activation, prime neutrophils, or instead stimulate macrophages or other cells. Thus, in PMNs, TNFα through a tyrosine kinase-mediated indirect step induces the serine phosphorylation of p47phox at the priming sites which are the same residues also phosphorylated by GM-CSF in a MAP-kinase-dependent manner [184].

In endothelial cells, TNFα has long been associated with Nox2 activation [185,186]. In these cells, TNFα induces an interaction of p47phox and TRAF [187] and activates PKC ξ which subsequently phosphorylates p47phox [188]. Thus, the TNF-induced endothelial ROS production is strictly dependent on p47phox [189]. In fibroblasts and smooth muscle cells, TNFα leads to Nox1 activation. Some groups report that this results in necrotic or necroptotic cell death through TRADD and Rac1 [190] whereas others link TNFα predominantly to inflammatory signaling. Given the differences in the upstream activation of Nox1, it was suggested that the adapter molecules Tks4 and Tks5 are specifically involved in the activation of Nox1 and Nox3 but not Nox2 or Nox4 in DLD1 colon cancer cells [191] in a process dependent on Src and Vav2 in HT29 human colon carcinoma cells [163]. This work, however, requires

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confirmation in other cell lines. Maybe not unexpected, the process of cell death itself has also been linked to Nox activation. After apoptosis induction by death receptor activation, riboflavine kinase facilitates assembly of the Nox1 complex at the plasma membrane of tumor cell lines [192]. Also, Nox1 is activated in response to DNA damage through a release of 14-3-3ζ proteins [193]. The subsequent ROS formation in both models is facilitated by Rac1 activation.

For smooth muscle cells, compartmentalization of inflammatory Nox1 signaling is relevant: Whereas the growth factor thrombin increases Nox1 activity at the plasma membrane through EGF receptor transactivation, TNFα signaling occurs through a dynamin-dependent endosomal pathway resulting in localized ROS production within the endosomes [194]. If Nox1 is missing in endosomes or if the charge compensation through chloride channels cannot take place, TNFα-induced inflammatory reactions and NFκB activation are attenuated [159]. Airway epithelial cells respond to TNFα stimulation with an increase in apoptosis rate. This occurs through stimulation of TNF receptor 1 and TRAF2 activation. Subsequently, Nox1 is activated which (presumably) through the consumption of redox thioredoxin leads to ASK1 activation. Subsequently JNK is phosphorylated and activated which then triggers apoptosis [195].

Also the family of toll-like receptors (TLRs) promotes Nox-dependent ROS production (beyond Nox4) and direct activation, priming, and induction of the oxidase have all been reported in almost 200 publications which cannot all be reviewed here (for more information, see [196]). For neutrophils it was suggested that TLR4-mediates activation of the interleukin-1 receptor-associated kinase-4 (IRAK-4) which phosphorylates p47phox in a PKC- and MAP kinase-independent manner acting mainly on p47phox Thr133, Ser288, and Thr356 [197]. In macrophages, a role of MyD88 through a stimulation of p38MAP kinase was suggested to mediate p47phox phosphorylation, ROS formation, and killing [198]. TLR7/8 agonists prime human neutrophils through p47phox phosphorylation and translocation [199]. In endothelial cells, TLR4 stimulation induced membrane translocation of the cytosolic subunits and activated Nox2 [200] whereas in cardiac muscle cells, TLR4 activates Nox1 [201]. Stimulation of TLR7 but not TLR3-mediated signaling was suggested to prime Nox2 in macrophages infected with influenza A virus [202], whereas TLR3 activation in response to viral infection of airway epithelial cells stimulates Rac1 and increases ROS production through Nox1 [203]. TLR2 in VSMC was reported to directly interact with Nox1 if over-expressed and loss of Nox1 prevented TLR2-induced ROS formation [204]. As if this was all not already very complex, others suggest that mitochondria are recruited to the phagosome in macrophages on TLR1, TLR2, and TLR4 stimulation and increase ROS formation [2]. Finally, much of the inflammasome response depends on "cotransmitters" such as ATP, which also increases Nox activation. For example, in J774 and H293 cells, stimulation of ATP-gated P2X7R receptors increases ROS formation through a pathway involving calcium and potentially Pyk2 or Src [205]. Collectively, it is certainly fair to state that TLR activation is linked to increased ROS formation. Due to the complexity of the TLR activating signals, the secondary reactions occurring, and the plethora of ligands of TLR receptors, there is still much to learn.

Growth factors

A large number of growth factors activating either tyrosine kinase receptors or G-protein-coupled receptors have been reported to activate NADPH oxidases and due to the sheer number of publications, this aspect cannot be fully covered here. In essence, however, similar activation pathways are always operative. Rac is activated either directly through G-protein-mediated GEF activation or in the case of tyrosine kinase receptors through PIP3-mediated GEF activation. Stimulation of phospholipase C isoforms facilitates protein kinase C activation, leading to p47phox phosphorylation, or p47phox is phosphorylated with PIP3-activated AKT or p21PAK. Phospholipase C-dependent release of IP_3 also results in increases in intracellular calcium. EGF receptor transaction is involved in Nox activation of some growth factors and calcium chelators usually block upstream Nox activation (for a more detailed review, see [206]) (Fig. 6).

Cytokines

The situation for cytokines is similarly complex. Some cytokines only prime the oxidase, whereas acute cytokine stimulation leads to a transient increase in Nox activation as documented for HGF [207,208]. As for other signals, this process involves PI3kinase and Rac and the recruitment of p47phox to the cytoskeleton and to lamellipodia [208]. Similar mechanisms are operative for erythropoietin [209], leptin [210,211], and IL-4 [212] and all involve

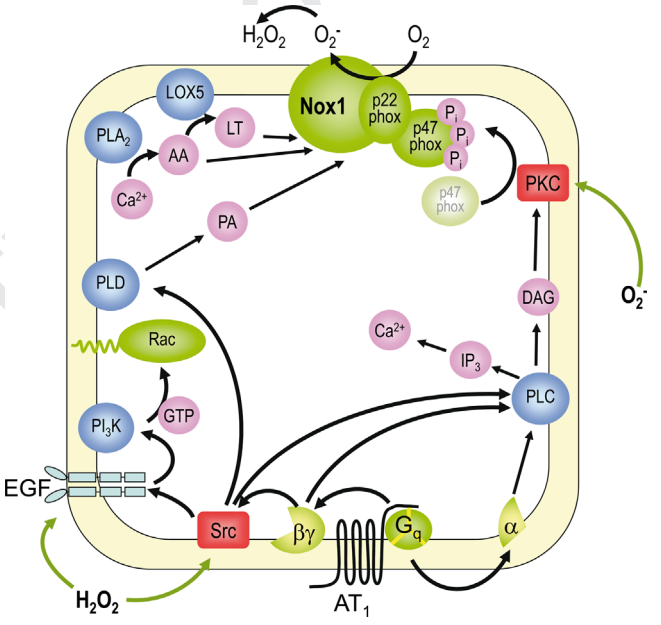

Fig. 6. Activation of Nox by growth factors. EGF, epidermal growth factor; AT1, angiotensin II type I receptor; PLC, phospholipase C; PLD, phospholipase D; PLA2, phospholipase A2; LOX5, 5-lipoxygenase; PKC, protein kinase C; PI3K, phosphatidylinositol 3-phosphate kinase; AA, arachidonic acid; LT, leukotrine B4; PA, phosphatidic acid; DAG, diacylglycerol.

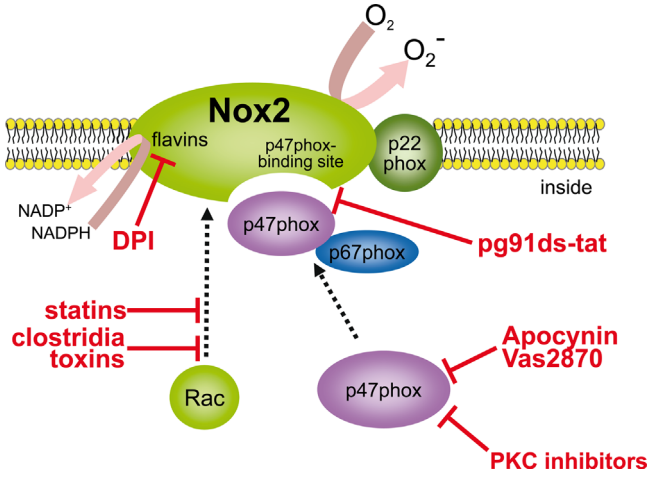

Fig. 7. Site of action of Nox2 inhibitors. PKC, protein kinase C; DPI, diphenylene iodonium.

PI3-kinase-mediated Rac and p47phox activation. In myeloid cells, G-CSF-mediated ROS production involves the pathway Lyn-PI3kinase AKT [213] and inhibition of AKT reduced p47phox phosphorylation and ROS formation in human neutrophils [57,58] (Fig. 7).

Signaling Lipids

Signaling lipids are a heterogeneous group of molecules which can act through G-protein-coupled receptors, TLRs, growth factor, and cytokine receptors. They may work as direct receptor ligands as in the case of sphingosine-1-phosphate, act as detergent, or change membrane fluidity. Particularly proatherogenic oxy-lipids have been studied in the context of Nox activation. These have the capacity to increase oxidase expression as shown for Nox4 and also to activate the oxidase through p47phox and Rac. Lysophosphatidylcholine activates ROS formation through a p47phox-dependent mechanism in VSMC [214] involving PKC [215] and Rac [216]. Similar findings were reported for lysophosphatidic acid release from platelets, which also activates Rac1 via Tiam1 [217,218]. Oxysterol stimulates the oxidase in macrophages through a PKC-dependent mechanism involving also p47phox translocation and arachidonic acid release, which is suggestive for a potential involvement of leukotrienes [219]. The LPC-mediated activation of the endothelial NADPH oxidase is sensitive to inhibitors of phospholipase A2, PKC and PI3-K [220], and leukotrienes and in particular leukotriene B4 in alveolar macrophages activate Nox via PKCδ [221]. Oxidized 1-palmitoyl-2-arachidonyl-glycerol-3-phosphocholine activates Rac1 and increases ROS formation [222]. Oxidized LDL has long been shown to activate NADPH oxidases [223–225] but also minimally oxidized LDL activates ROS formation in macrophages through a pathway involving TLR4, MyD88. The subsequent activation of Syk stimulates activation of PLCγ1 PKC and eventually Nox2 [226]. Sphingosine-1-phosphate contributes to fMLP-induced Nox2 activation by controlling intracellular calcium [227] but in fibroblasts [228] and arteries [229] S1P also activates NADPH oxidases. It should be noted that the authors' groups did not find a S1P-induced Nox activation [230] and even suggest Rac inhibition [231], which is, however, in our hands not the case (R. Brandes, unpublished observation, 2012).

Hypoxia/hypoxia reoxygenation

Obviously, hypoxia and anoxia are rather reductive situations with a shortage of substrate for ROS formation—oxygen. It should, however, be noted that in abscesses and in necrotic regions which are invaded by PMNs and macrophages, Nox2, with a fairly low K_m of 36 μmol/L for oxygen is still somewhat active [99]. This paragraph, however, rather addresses the question whether hypoxia/reoxygenation is a stimulus for oxidase activation. It is fair to say that for reoxygenation this question has been solved as Rac and PI3-K activations occur and subsequently activate Nox1 and Nox2 [232–234]. A possible mechanism is that the cellular depolarization occurring during I/R activates Rac [59]. The process is associated with increased tyrosine phosphorylation whereas hyperpolarization prevented Rac activation and reduced ROS production [235]. Whether mitochondria or Nox are the primary source of ROS under this condition is unclear. It is also not known whether a cross talk between the two systems mediates the finally deleterious reperfusion injury. For hypoxia, the situation is less clear also because of the lack of reliable ROS detectors. It was suggested that hypoxia increases the ROS formation by endothelial cells in a Nox-dependent manner, but the underlying mechanism is still unclear [236] and potentially, this is an endothelial cell-specific phenomenon.

Cyclic moderate nontoxic hypoxia as present in sleep apnea is also associated with Nox-derived ROS production which is attributed to Nox2 [237,238] and also a p22phox polymorphism in patients is associated with sleep apnea [239]. Whether Nox2 is induced under this condition is controversial [237,240] but Nox4, as being hypoxia inducible, is known to increase [241]. As H_2O_2 is produced in proportion to the pO2 by Nox4 [101], it is attractive to speculate that particularly during cyclic hypoxia, Nox4 contributes to H_2O_2 production during the normoxic phase [242].

Diabetes/high glucose/hyperinsulinemia

Dysregulation of glucose metabolism is linked to increased ROS production through multiple pathways. Elevated glucose activates PKC isoforms and PI3-K [243–245] and high glucose activates NADPH oxidases in a PKC- [246], Rac1- [247], and p47phox-dependent manner [248]. In diabetes mellitus II, however, other mechanisms also contribute to Nox activation such as changes in AMP-regulated kinase activity and hyperinsulinemia. Although the mechanisms of insulin-induced Nox activation are complex, a role for Nox2 as well as for Nox4 has been suggested. In 3T3-L1 adipocytes dominant negative Nox4 blocks Nox4-induced ROS formation [167], but so far the data were not confirmed in knockout mice. In skeletal muscle cells, insulin induces an increase in H_2O_2 through a PKC-dependent pathway which was blocked by a Nox2-inhibitory peptide as well as by p47phox knockdown, suggesting an involvement of Nox2 [249,250]. Also advanced glycation end products (AGEs) increase p47phox phosphorylation and subsequent ROS formation in an immortalized corneal epithelial cell line [251]. In bovine retinal endothelial cells, AGEs induce PKCβ and p47phox translocation as well as ROS formation [252]. Diabetes also facilitated neutrophile priming. In HL-60 cells exposed to high glucose, this was mediated by Erk1/2 but not p38 MAPK [253] and serum of type II diabetics induces endothelial E-selectin expression in cultured HCAEC through a pathway involving ROS production [254]. Collectively, these data confirm that dysregulation of the glucose homeostasis through multiple mechanisms increases Nox-derived ROS. Nox enzymes are, however, not the only source of ROS under this condition and mitochondria and uncoupling of endothelial NO synthase also importantly contribute to the oxidative stress.

Cellular mechanic stimuli

Mechanic stimulation of cells increases Nox activity and extensive literature has been published on this topic. The reader is kindly referred to a review exclusively covering this complex matter [255]. Mechanostimuli release Nox-activating factors from the cellular environment and induce receptor-mediated Nox activation, for example, by EGF transactivation [256]. Moreover, cellular mechanosensors couple to Rac, PI3-K, PKC, and p47phox and thereby directly facilitate Nox activation [257]. High pressure increases Nox activity in vessels through a PKC-dependent mechanism [258] and in vascular smooth muscle cells, this effect was absent after genetic deletion or inhibition of p47phox [259]. Osmo stress results in the activation of PKC ζ, ceramide formation, and p47phox phosphorylation followed by ROS production in hepatocytes [260]. Similarly, osmotic swelling by a pathway involving protein kinase C ζ, NMDA receptor, and calcium stimulates Nox2 in a p47phox-sensitive way in astrocytes [261]. Interestingly, the adhesion state of leukocytes via integrin signaling has a strong impact on Nox activation. In contrast to its effects in endothelial cells, TNFα in leukocytes does not per se stimulate p47phox/Nox2 interaction but requires prior adhesion of the cells. Adhesion through integrin signaling appears to allow PKCδ translocation to p47phox, which results in ERK1/2 recruitment [262].

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Collectively, again the complexity of cell type and stimulus illustrates that some uniform pathways of Nox activation are present but detailed analysis reveals that responses are context and cell dependent.

Inhibition of Nox activation

It is obvious to assume that in addition to pathways promoting Nox activation also those limiting Nox-dependent ROS production have evolved and one of them is the SUMO system. SUMO1 acts as endogenous inhibitor of Nox1 and Nox5 in HEK293 cells, hVSMC [263], and neutrophils [264], although the proteins and residues subjected to sumoylation are unknown. Another aspect of activity control is proteolytic cleavage of the cytosolic subunits or cytosolic parts of Nox2 or even Nox4. Activation of caspase-1 can perform this task on NLRP3 inflammasome activation in phagocytes [265].

Phosphorylation signals

The activity-regulated inhibitory endogenous systems act through phosphorylation signals and the AMP-activated protein kinase (AMPK) and the protein kinase A (PKA) are most important. Recently particularly AMPK gained significant interest as AMPK knockout mice exhibit oxidative stress and vascular dysfunction [266,267]. AMPK has an inhibitory effect on Nox activity and reduced the PMA- and fMLP-stimulated ROS formation of human neutrophils [268]. AMPK stimulation with 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide reduces p47phox phosphorylation and simultaneously increases p38 and JNK phosphorylation [268]. The AMPK activators metformin and rosiglitazone induce PKC inhibition and reduction in ROS formation during high glucose [244,269].

Several publications suggest that cAMP/PKA are negative regulators of Nox activity. Vasoactive intestinal peptide reduces pancreatic ROS formation by increasing cAMP/PKA activity [270]. cAMP is the product of adenylyl cyclase, which is stimulated by Gs in response to prostaglandins and also in response to β receptor stimulation. The prostacyclin analogue iloprost prevents the inflammation-induced Nox1-mediated induction of thromboxane A synthase [271]. Microsomal prostaglandin synthase-1 derived prostaglandin E2 is also able to block Nox activation in response to angiotensin II in mouse VSMCs [272] and prostaglandin E2 suppresses bacterial killing in alveolar macrophages by blocking p47phox phosphorylation and translocation [273]. Resolvin D1 through increasing cAMP attenuated the efferocytosis-induced p47phox translocation, phosphorylation, and ROS formation in RAW263.7 cells [274]. Interestingly, different than cAMP, increasing cGMP does not appear to limit Nox activation in human neutrophils [275], as revealed from studies with the sGC activator YC-1.

Of more narrow interest are a few pathways which are nevertheless worth noting. The Scr homology phosphtase-1 (SHP-1) is activated in response to angiotensin II receptor type 2 activation and limits angiotensin II-induced ROS formation [276]. Similarly, stimulation of the endothelin-1 B receptor (ETB1) blocks the phosphorylation of Pyk2 and Rac1 and thereby inhibits Nox activity [277]. In microglia activated by LPS, TGFβ inhibits the Nox2-dependent ROS formation by preventing the Ser345 phosphorylation of p47phox [278]. Activation of extranuclear estrogen receptor alpha (ERα) through phosphorylation of Akt and subsequently Rac1 leads to inhibition of Nox2 in hippocampal neurons [279].

Gaseous transmitters as inhibitors

The three gaseous transmitters nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S) all mediate protective signaling. As Nox enzymes contain two heme in their transmembrane region as well as cysteins, they could be considered as potential targets for these molecules, although most work has been done with respect to CO. In VSMC heme oxygenase-1-derived CO but not the HO-1 products biliverdin or bilirubin blocks VSMC migration as a consequence of Nox1 inhibition [280]. In endothelial cells, Nox enzymes might be inhibited through a NO-triggered induction of heme oxygenase-1 and the subsequent production of bilirubin was suggested to block Nox assembly; CO, however, was not tested [281]. In response to TNFα stimulation, CO production was suggested to inhibit Nox4 and to mediate an antiapoptotic effect in brain microvascular endothelial cells, but this also was not directly shown [282]. Nevertheless, it has not yet been studied whether CO directly binds to the heme irons and thus these data must be interpreted with caution. Also the activity of Nox4 is attenuated by heme oxygenase 1 [152]. In VSMCs, H_2S reduces Nox1 expression and also attenuates Rac1 activity and these effects appear to be mediated by PKA activation [283]. Finally, high concentrations of NO attenuate NADPH oxidase activity by S-nitrosation of p47phox [284]. NO, however, also inhibits the p47phox-independent Nox5 by reversible S-nitrosation under physiological and pathophysiological conditions. Particularly nitrosation of C694 dramatically lowered Nox5 activity, and the effect could be attenuated by GSNO reductase and thioredoxin1 [285].

Nox inhibitory peptides

On the basis of peptide walking it was identified that peptides that block the interaction of the cytosolic subunits with Nox2 can act as potent and highly selective Nox inhibitors. The principle was refined by P. Pagano and his group to yield isoform selective, cell-permeable inhibitors for Nox1 and Nox2 [286,287]. Nox2ds-TAT selectively inhibits Nox2 [288] and recently also an inhibitor which selectively blocks Nox1 by mimicking NoxA1 (NoxA1ds) with nanomolar efficiency was released [35]. Importantly, compatible with the lack of cytosolic interacting proteins, in a reconstitution overexpression system, screening of a peptide library to inhibit Nox4 did not yield any hits in the B loop and C-terminal end of the protein [149]. Currently, Nox2-ds-TAT and NoxA1ds are the most isoform selective and the only specific Nox inhibitors. Moreover, the mode of action of these compounds is indisputable but peptide inhibitors bear the obvious limitation that their duration of application is limited and that they must be injected if used in vivo.

Small molecule inhibitors

Considering the limitation of peptide inhibitors, small molecule inhibitors are currently being developed by several groups and companies. The availability of several of these inhibitors is, however, limited as they are not yet commercially available or recently published experimental compounds. Some drugs have Nox inhibitory activity like the betablocker nebivolol [289], the benzodiazepam antagonist naloxone [290], and the anaesthetic propofol [291], but whether their Nox inhibitor capacity is of importance in humans is unclear. Other reported inhibitors are antioxidants such as ebselen [292], plumbagagin [293], and fluvene-5 [294], making it difficult to attribute their effects specifically to Nox inhibition. Some other inhibitors have recently been reported but it is currently too early to advocate them. The plant component celastrol acts as inhibitor of Nox activation by blocking the interaction of Nox1 and Nox2 with the cytosolic components [295]. Acetylphenothiazine is suggested to be a Nox1

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inhibitor [296] but inhibitors with better Nox1 selectivity have recently been reported with ML171, being the most effective and selective one [297–299]. Also a screen for Nox4 inhibitors was published but almost nothing is yet known about these compounds [300].

Apocynin

A particularly interesting Nox inhibitor is apocynin. The compound itself is not a Nox inhibitor but rather a prodrug with antioxidant actions [301]. On activation by peroxidases, apocynin forms a radical which is thought to form adducts with p47phox [302] and also apocynin multimers form, which are reported to act as Nox inhibitors through an unknown mode of action [303]. Importantly, apocynin activation results in the formation of a radical and thus apocynin can induce oxidative stress in the cell [304] and reduces the level of glutathione. Thus, in cells with low Nox but significant peroxidase activity, apocynin increases rather than decreases ROS level [305]. Further nonspecific effects of apocynin are the inhibition of agonist-induced platelet activation in a Nox-independent manner [306] and the inhibition of Rho kinase [307]. Thus, data obtained with apocynin must be interpreted very cautiously.

Diphenylene iodonium (DPI)

DPI blocks many flavoenzyme. DPI is therefore a potent but totally nonspecific inhibitor of all Nox enzymes and also inhibits all other flavoenzymes. It blocks mitochondria [308] and therefore stimulates glycolysis and activates AMPK [309]. It also inhibits cytochrome P450 monoxygenases [310] and NO synthases [311–313]. This array of effects also makes DPI incompatible with in vivo use.

VAS2870

VAS2870 was initially reported as a selective Nox inhibitor [314]. Vas2870 does not inhibit Nox4 if overexpressed in HEK293 cells but inhibits Nox2 as well as Nox1 if transfected together with their cytosolic activator proteins (R.P. Brandes, unpublished observations). This pharmacological profile is compatible with the discussed mode of action: VAS2870 directly modifies thiols like the ones in p47phox but it also modifies the ryanodine receptor R1 through thiol-alkylation and therefore significant off-target effects are present for this compound [113].

Toward intelligent Nox inhibition

Nox NADPH oxidases elicit numerous effects on signal transduction [315] and importantly, most of them appear to be physiologically meaningful already on first sight. We must acknowledge that Nox enzymes have multiple important physiological functions in the cell and therefore Nox inhibition may perturb normal physiology in unpredictable ways. For example, in the cardiovascular system all Nox enzymes contribute to physiological healing effects and maintain vascular function [316], and inhibition of this physiological Nox signaling might result in vascular dysfunction, acceleration of atherosclerosis, and vascular complications. Paradoxically, the same enzymes are induced and overactive during cardiovascular disease development and their inhibition or deletion limits disease progression in animal experiments and even in a few human studies [316]. Direct Nox inhibition might be one way to address enzyme overactivation during disease development. A potential alternative is to interfere with the upstream pathway of Nox activation or to lower the pathologically increased Nox expression. Angiotensin II receptor blocks and statins have convincingly demonstrated this approach to be safe and to reduce—at least in the vascular system—oxidative burden without (Nox-related) side effects [317].

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