The NOX toolbox: validating the role of NADPH oxidases in physiology and disease

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Abstract Reactive oxygen species (ROS) are cellular signals but also disease triggers; their relative excess (oxidative stress) or shortage (reductive stress) compared to reducing equivalents are potentially deleterious. This may explain why antioxidants fail to combat diseases that correlate with oxidative stress. Instead, targeting of disease-relevant enzymatic ROS sources that leaves physiological ROS signaling unaffected may be more beneficial. NADPH oxidases are the only known enzyme family with the sole function to produce ROS. Of the catalytic NADPH oxidase subunits (NOX), NOX4 is the most widely distributed isoform. We provide here a critical review of the currently available experimental tools to assess the role of NOX and especially NOX4, i.e. knock-out mice, siRNAs, antibodies, and pharmacological inhibitors. We then focus on the characterization of the small molecule NADPH oxidase inhibitor, VAS2870, in vitro and in vivo, its specificity, selectivity, and possible mechanism of action. Finally, we discuss the validation of NOX4 as a potential therapeutic target for indications including stroke, heart failure, and fibrosis.

Keywords siRNA · Antibodies · NADPH oxidase inhibitor · NOX4 · VAS2870 · NOX4 knock-out

Oxidative stress: the need for validated targets and therapeutic specificity

Reactive oxygen species (ROS) have long been suspected as being ‘bad guys’. They are frequently associated with the development and progression of chronic, degenerative, cancerous and inflammatory diseases. Indeed an excess of ROS, i.e. oxidative stress, caused by an imbalance between ROS production and their removal by antioxidant systems, may be a common underlying pathogenic mechanism in these diseases. With the recent additional description of possible roles of ROS in diverse physiological signaling processes another form of imbalance deserves attention, i.e. reductive stress—the excess of reducing agents in a cell that leads to shortage of ROS. These and other phenomena may explain the poor outcomes of antioxidant therapies in clinical studies where even deleterious effects of untargeted antioxidant treatment have been reported [2–10]. Rather than attempting to systemically scavenge ROS, it may be more effective to specifically target the different enzymatic sources of pathophysiologically relevant ROS. Nevertheless, until this has resulted in clinical benefits, the oxidative stress hypothesis remains unproven.

Several ROS producing enzyme systems exist, including xanthine oxidase [11], the mitochondrial respiratory chain [12], lipid peroxidases [13], cytochrome P450 enzymes [14], and uncoupled endothelial NO synthase [15]. However, these enzymes produce ROS secondary to their damage, which can be proteolysis but is often caused by oxidative stress itself [11, 15]. Thus, there would still be
the need to identify this primary source of oxidative stress. The only enzyme family known to produce ROS as their primary and sole function are NADPH oxidases. These multi-protein complexes are comprised of a catalytic, transmembrane-spanning subunit (NOX), as well as several structural and regulatory proteins localized in both the membrane and the cytosol.

The NADPH oxidase family

We are only beginning to understand the enzyme family of NADPH oxidases, their players and their interaction. The NOX family consists of seven members, NOX1–5, and two dual oxidases (Duox), Duox1 and Duox2. Of those, NOX1, 2, 4, and 5 have been implicated in vascular diseases, on which we focus in this review. All NOX isoforms have six trans-membrane spanning alpha helices with cytosolic N- and C-termini. They are differentially expressed and regulated in various tissues and have different subcellular localizations, and even different ROS products, i.e. superoxide versus hydrogen peroxide (reviewed in [16]). NOX1, NOX2, and NOX5 appear to produce mainly superoxide NOX4, mainly H2O2 [17]. All NOX isoforms have been reported to bind to one or more membrane and/or cytosolic proteins. p22phox appears to be a general binding partner for NOX1-4 in the membrane. NOX1 and 2 also bind the small GTPase, Rac. Moreover, NOX1 binds the cytosolic subunits, NOX organizer 1 (NOXO1) and NOX activator 1 (NOXA1), and NOX2 binds the respective homologues, p47phox and p67phox, and also the cytosolic protein, p40phox [18, 19]. NOX4 was reported to bind to the polymerase (DNA-directed) delta-interacting protein 2 (PolDip2) [20]. In addition to these established NOX binding partners, the tyrosine kinase substrate with 4/5 SH3 domains (Tks4/5) [21, 22], and protein disulfide isomerase (PDI) were recently suggested to bind to both NOX1 and 4 [23]. Upon overexpression in cells, the C-terminus of NOX5 was shown to interact with Hsp90, which may also bind to NOX1 and 2 [24]. However, the physiologic relevance of

![Fig. 1](https://example.com/fig1.png)

**Fig. 1** The vascular NOX isoform-based NADPH oxidase complexes. Cell or subcellular compartment membranes are shown in gray, core proteins in yellow, activator binding proteins in green and organizer binding proteins in blue. All the NOX isoforms shown are membrane proteins and are localized in the plasma membrane (PM). Additionally, NOX1 was found at the plasma membrane in caveolae [147], NOX2 in membranes of phagosomes, and NOX4 in mitochondrial [182] and ER-membranes [191], as well as in the nucleus [97]. Little is known about subcellular localization of NOX5 other than the plasma membrane, but a localization at the ER membrane has been reported [29, 192]. NOX1, NOX2, and NOX4 are associated with p22phox, but only NOX1 and NOX2 are regulated by the small GTPase Rac. For its activation, the NOX1 enzyme complex requires the assembly of NOX organizer 1 (NOXO1) and NOX activator 1 (NOXA1), but also forms complexes with p47phox and p67phox (not shown). The NOX2 enzyme complex requires binding of p47phox, p67phox, and optionally p40phox that can further support the activity. In contrast to NOX1 and NOX2, NOX4 and NOX5 do not depend on any of the ‘classical’ cytosolic NADPH oxidase subunits. Recently, the protein polymerase (DNA-directed) delta-interacting protein 2 (PolDip2) was identified to bind and to increase the activity of NOX4. Further, protein disulfide isomerase (PDI) [23] and a p47phox analogue tyrosine kinase substrate with 4/5 SH3 domains (Tks4/5) have been reported to bind and activate NOX1 and NOX4 [21, 22]. NOX4 is the only isoform that produces hydrogen peroxide instead of superoxide [17]. The NOX5 protein contains four N-terminal calcium-binding sites that regulate activation of the enzyme. Activity of NOX5 can be further supported by the binding of Hsp90 or Calmodulin to the C-terminus of the protein [24].
| NOX isoform | Species | Sequence | Degree of NOX4 down-regulation (% of ctr.) | Ref./source | Comment |
|-------------|---------|----------|------------------------------------------|-------------|---------|
| NOX4        | Bovine  | 5'-AAGACCTGGCCAGTATATATT-3' | n.q. (protein) | [94] |         |
| NOX4        | Human   | 5'-GAGAACAGACCUGACUAUG-3' | 75–85 % (protein), ~ 10 % (mRNA) | [95, 96] | Tested vs. NOX1 and NOX2 |
| NOX4        | Human   | 5'-GUUCUUAACCUCAGAUGUGATT-3' (sense); 5'-UGCAUGGAGGCUUAAAGAATT-3' (antisense) | n.q. (protein, mRNA) | [97] | Base error according to database sequence |
| NOX4        | Human   | 5'-UUAAUUGAUAUGAAGAGCUUGAU-3' (sense); 5'AUCACAGCCUCUCAUAUGCAUA-3' (antisense) | n.q. (mRNA) | [98] |         |
| NOX4        | Human   | 5'-GUCAACACCGCUUGACCGCdtdt-3' (sense); 5'-GUCAACACCGCUUGACCGCdtdt-3' (antisense) | 20 % (mRNA) | [99] |         |
| NOX4        | Human   | Target sequence 5'-CAG TGA ACT ATA GTG AAC ATT TCC T-3' | 40 % (mRNA) | [100] | vs. NOX2 |
| NOX4        | Human   | Pool of 4: (1) ACUAGAUUACUUCUGGA; (2) GAAAUUAACCCAAAGCUGUA; (3) GGCUAGGAUUGUGUCUA; (4) GAUCACAGCCUCUCAAU | n.q. (mRNA) | Dharmacon [101] |         |
| NOX4        | Human   | Targets exon2 | 40 % (mRNA and protein) | Ambion [102, 48] | ID #118807 |
| NOX4        | Human   | 5'-XCCACCAACCACACCACATT-3'; 5'-AAUGUGGUUG GUGGGUGUGTT-3' | n.s. | [103] |         |
| NOX4        | Human   | n.s. | n.q. (protein) | Qiagen [104] | Hs_NOX4_1 and Hs_NOX4_2 predesigned |
| NOX4        | Human   | 5'-CAAGAACATTCCTCATATT-3' & 5'-ACTTTGTGGAACTGAAATG-3' | n.q. (mRNA) | [105] |         |
| NOX4        | Human   | Mixture of: (1) 5'-AAAGCAGGACUCU UCAUGGA GAGCCA-3' (sense); 5'-UGGCUCUCCAUGAUGGUC UGGCUUU-3' (antisense); (2) 5'- GCAUCUGUUUCUAAACCUCA-3' (sense); 5'-UGAGGUUAAGAGCAAGAUCG-3' (antisense); (3) 5'-CCAGGAGAUUGUGGUAA-3' (sense); 5'- UUAUCCAACAAUCCUG-3' (antisense); (4) 5'-CAGUAAGACUUUGGUGGAACUAU-3' (sense); 5'- AUUCAGGAUCAAACAAACUUACUG-3' (antisense) | ~ 40 % (mRNA) | [106] | Sequences not present in NOX1, NOX2, NOX3, and NOX5 |
| NOX4        | Human   | 5'-AGACCUGGCCAGUAUUA-3' | ~ 30 % (mRNA) | [107] |         |
| NOX4        | Human   | n.s. | ~ 38 % (mRNA) n.q. (protein) | [108] | Tested vs. NOX1, NOX2, NOX3 |
| NOX4        | Human   | NOX4, 5_-CCU CUU CUU UGU CUU CUA C dTdT-3_ corresponding to nucleotides 585–603 | ~ 33 % (mRNA) | [109] |         |
| NOX4        | Human   | 5'-CGAGAUGAGAAGUCCUAGAAAdCdtdt-3' (sense); 5'-UUCAUGGAAUCCUAUCUGCdtdt-3' (antisense) | ~ 25 % (mRNA) | [90] |         |
| NOX isoform | Species | Sequence | Degree of NOX4 down-regulation (% of ctr.) | Ref./source | Comment |
|------------|---------|----------|------------------------------------------|-------------|---------|
| NOX4       | Human   | 5'-GGUACAGCUGGAUGUUGAC-3' | 50 % (mRNA) n.q. (protein) | [92]         |         |
| NOX4       | Human   | 5'-AAACGGGGCAAGGUAUCCCAG-3' | ~45 % (protein) n.q. mRNA | [110, 111]  |         |
| NOX4       | Human   | 5'-GTCAACACATCCAGCTGACCdTdT-3' | n.q. (mRNA) | [112, 113] |         |
| NOX4       | Human   | (1) 5'-GATCCGAGACACTTCCATATTACTICAAGAGAATTAATA | n.q. (mRNA and protein) | [114] |         |
| NOX4       | Human   | (2) 5'-GATTCCGAATTCGTTGGAAACCCAG-3' | n.q. (protein) | [93] | (1) and (2) not efficient, (4) most efficient |
| NOX4       | Human   | (3) 5'-GAAUUACAGUGAAGACUUU-3' | 50 % (mRNA) n.q. (protein) | [115, 116] | NOX5 not affected |
| NOX4       | Mouse   | 5'-GAC CUG ACU UUG UGA ACA UTT-3' (sense); 5'-AUG UUC ACA AAG UCA GGU CTT-3' (antisense) | 30 % (NOX activity, protein, mRNA) | [47, 117] | Tested vs. NOX1 recommended |
| NOX4       | Mouse   | 5'-GGCCAACGAAGGGGUUAAAACACCUC-3' | n.q. (mRNA) | [118, 119] |         |
| NOX4       | Mouse   | 5'-GGGUAAGAAGCAAGCUCACACAC-3' | (mRNA) | [119] |         |
| NOX4       | Mouse   | Mix of 3 siRNAs: 5'-CCAUUUGCAUCGAUACUAAU-3'; 5'-CAGACUCUCUCUAAGGUGU-3'; 5'-GUAGGUAGGAGGCUGUGAUC-3' | 40 % (mRNA) | Santa Cruz [120] |         |
| NOX4       | Mouse   | Target sequence: 5'-CAGGAGAAATAATTAAAGCTTTA-3' | n.s. | [121] |         |
| NOX4       | Mouse   | 28-kDa NOX4 (5'-AATTTGTTGGGCTGCTACTGA-3' (sense); UGUUGGCCUCUGUACUGAdTdT (antisense), UCAGUAGGACAGCCCAACAdTdT and full-length 65 kDa and 28 kDa (5'-AACGAAGGGGTGTTAACACCTC-3' and 5'-AAAAGCAAGACTCTACATAC-3') | 80 % (mRNA), 60 % (protein) | [43] |         |
| NOX4       | Mouse   | n.s. | 18 % (mRNA) | Santa Cruz [122] | vs. NOX2 |
| NOX4       | Mouse   | n.s. | n.q. (protein) | Ambion [123, 124] | ID #184259 and #184261 |
| NOX4       | Mouse   | Pool of 3–5 siRNAs | n.q. (mRNA and protein) | Santa Cruz [125] | # sc-41587 |
| NOX4       | Mouse   | (1) 5'-AACGAAGGGGTGTTAACACCTC-3'; (2) 5'-AAAAGCAAGACTCTACATAC-3' | n.q. (protein) | [126] |         |
| NOX4       | Mouse   | 5'-GGUUACAGCUCUACCAUCUAC-3' (sense); 5'-GUAGGUAGGAGGCUAGUACC-3' (antisense) | n.q. (protein and mRNA) | Dharmacon [93] | In vivo treatment |
| NOX4       | Pig     | n.s. | 50-60% (protein) | Dharmacon [127] | Tested vs. NOX2 |
these new potential binding partners for NOX function needs to be further analyzed (Fig. 1).

With respect to activity regulation, there are fundamental differences between the individual NOX catalytic subunits. Most seem to be dynamically switched on and off by either regulatory subunits (NOXA1 for NOX1 [25–27], p67phox for NOX2 [28], and calmodulin for NOX5 [29, 30]) or intramolecularly by the N-terminal EF hands that bind free intracellular calcium (NOX5 and Duox1/2 [31]). In contrast, NOX4 is constitutively active, and modulation of its expression may thus be a major activity regulator.

The tools to validate the role of NADPH oxidase in health and disease

During the validation of the involvement of a protein in a biological process or disease mechanism pharmacological
inhibition or genetic deletion are frequently applied. In addition, specific antibodies are required to confirm the expressional regulation of NOX in a given cell or subcellular compartment. With respect to NOX biology these tools include genetic knock-out [32–35] and transgenic animals [32, 36, 37], pharmacological inhibitors, and siRNAs (see Table 1).

NOX knock-out mouse models

NOX2 knock-out (KO) mice in which exons 2 and 3 are deleted are commercially available [38], and no other NOX2 KO model has been published. Two identical NOX1 KO mice carrying a deletion of exons 3–6 have been published showing a mild hypotensive phenotype and attenuated angiotensin II-induced hypertension [39, 40]. Unfortunately, no western blot data using tissues of these mice to confirm the absence or size of a possibly residual NOX1 protein have been published. An N-terminally truncated or alternatively spliced NOX1 protein may still be expressed [41]. However, it is unlikely that NOX1 splice variants lacking the binding sites for regulatory subunits have any ROS-producing activity. With respect to NOX4, there is more variety, and four NOX4 KO mouse models have been published to date (Fig. 2). All differ in the genetic strategy that was applied to generate them, i.e., different exons were deleted (exons 1/2, exon 4, exon 9, or exons 14/15) and constitutive, cell-specific or inducible cre/lox systems were used. In future, this may also help to elucidate the role of alternative splicing in mouse NOX4 biology [32–35]. Indeed, the possibility exists that, at least in some tissues, the deletion of an early exon may lead to truncated but active NOX4 variants and thus residual NOX4 activity. Interestingly, an analogue to the human NOX4 splice variant D [42] lacking exons 3–11 of murine NOX4 has been found in kidney and colon. Importantly, this 28-kDa NOX4 isoform (Fig. 2c) was still capable of producing ROS, and the authors could blunt this activity by selective siRNA silencing of this particular isoform [43]. This observation is supported by the findings that the isolated NOX4 dehydrogenase domain is still able to reduce substrates like certain artificial dyes [44]. Although not shown directly for NADPH oxidases, it is known that flavin-binding domains are able to reduce oxygen, thus forming superoxide [45, 46]. Accordingly, the residual NADPH- and flavin-containing protein seems to be sufficient to catalyze ROS formation. Only in mice containing a deletion of either exon 9 (FAD binding site) or 14/15 (NADPH binding site) is it unlikely that any residual NOX4 protein could still produce ROS. It is discussed in the field that potential shortened inactive NOX4 proteins present in exon 9 or exons 14/15 deletions exert dominant negative or positive effects on other NOX isoforms (e.g., NOX1 and NOX2) or NOX binding proteins. For example, in the absence of NOX4, more free p22phox may be available to interact with NOX1/2. Such mechanisms could affect both the expression and activity of other NOX isoforms. However, protein levels of other NOX isoforms have not been reported to be altered in NOX4 KO mice [33]. Further, if the activity of other NOX isoforms would be influenced these mice would then be expected to show a mixed phenotype of NOX4 and NOX1 and/or NOX2 KO mice, e.g., reduced blood pressure and angiotensin II-induced pressure response (NOX1; [39, 40]) or impaired oxidative burst activity of circulating neutrophils (NOX2; [38]). The neutrophil phenotype remains to be analyzed. A dominant negative regulation of other NOX isoforms in other cell-types of NOX4 KO cannot be completely ruled out unless studied. The lack of an effect on blood pressure by NOX4 deletion in mice [33] argues against such a hypothetical mixed NOX1/4 phenotype.

Transgenic NOX4 overexpressing mouse models

Parallel to the NOX4 KO mice, three different transgenic NOX4 (tgNOX4) overexpressing mice have been published, two of a cardiomyocyte-specific manner [32, 36] and the most recent in an endothelial-specific manner [37]. Surprisingly, the endothelial tgNOX4 mouse had a lower systemic blood pressure compared to littermate wild-type mice, which does not match the vascular phenotype of any of the NOX4 KO mice, which are all reported to have unchanged blood pressures [32–34]. Similar to the discussion above on bystander effects on other NOX isoforms in NOX4 KO mice, NOX4 overexpression may also affect both expression and activity of NOX1/2. For example, less p22phox may be available to interact with NOX1/2. However, NOX1 was below detection limits in aortae from both wild-type and tgNOX4 animals, and NOX2 levels were unchanged [37]. Thus, dominant negative effects of a transgenic expression of NOX4 on other NOX isoforms cannot be excluded, but based on all available data are unlikely. The discrepancy in blood pressure might be due to non-physiologically high levels or different subcellular localization of the overexpressed NOX4 compared to endogenous NOX4, a general problem of transgenic overexpression models. A similar subcellular localization of tgNOX4 and endogenous NOX4 was shown in cardiomyocytes [32], but no immunofluorescence data in the endothelium have been published up to date.

siRNA mediated knock-down of NOX4

There are an increasing number of reports using siRNAs approaches directed against NOX4 (Table 1). Unfortunately, only a few of those siRNAs have been properly
### Table 2 Antibodies: a selection of published antibodies raised against NOX proteins and their main characteristics (if known)

| NOX isoform | Species | Antigen | Type | Size of detected protein in WB (kDa) | Ref./source | Comment |
|-------------|---------|---------|------|--------------------------------------|-------------|---------|
| NOX1        | Human   | aa 480–493 | pAb rabbit | n.s. | [132] | |
| NOX1        | Human   | aa 544–556 | pAb rabbit | 63 | [133, 134] | |
| NOX1        | Human, rat, mouse | aa 545–561 | pAb rabbit | 134 | [33, 52, 87, 135–138] | Recommended |
| NOX1        | Human, rat, mouse | Various | pAbs | Commercial | Not recommended |
| NOX1        | Rat | aa 543–558 | pAb rabbit | 75 | [130] | |
| NOX2        | Human, rat, mouse | aa 548–560 | pAb rabbit | 53, 91 | Upstate Technologies, BD Biosciences | Ab from upstate recommended for WB, Ab from BD for IF |
| NOX4        | Human   | aa 84–101 | pAb rabbit | 65 | [5, 52, 138] | |
| NOX4        | Human | aa 88–102 | pAb rabbit | ~ 70 | [110, 139–142] | |
| NOX4        | Human | aa 139–154 and 564–578 | pAb rabbit | 62 | [95] | |
| NOX4        | Human | aa 140–153 | pAb rabbit | ~ 70 | [143] | |
| NOX4        | Human | aa 222–241 | mAb | ~ 58 and 65 | [17, 50] | |
| NOX4        | Human | aa 251–266 | pAb rabbit | ~ 65 and 90 | [78, 144] | |
| NOX4        | Human | aa 256–273 | pAb rabbit | 65 | [145, 146] | |
| NOX4        | Human | aa 320–428 (recombinant peptide) | pAb rabbit | 65, 80 | [20, 93, 103, 105, 108, 115, 126, 136, 147–170] | |
| NOX4        | Human | aa 389–416 | mAb | ~ 58 and 65 | [50] | |
| NOX4        | Human | aa 392–398 | mAb | ~ 58 and 65 | [50] | |
| NOX4        | Human | aa 406–578 | pAb rabbit | n.s. | [97] | |
| NOX4        | Human | aa 499–511 | pAb rabbit | 66 and 72 | [97, 171] | |
| NOX4        | Human | aa 500–550 | mAb rabbit | 66 | [53] | |
| NOX4        | Human | aa 553–573 | pAb rabbit | 70 | [172] | |
| NOX4        | Human | aa 556–568 | pAb rabbit | 65 | [17, 32, 33, 47, 51, 87, 125, 173] | Recommended |
| NOX4        | Human | aa 556–569 | pAb rabbit | 64 | [42, 92] | |
| NOX4        | Human | aa 558–578 | pAb rabbit | n.s. | [105] | |
| NOX4        | Human | aa 559–578 | pAb rabbit | 66 + 2 bands >94 | [97, 98, 101, 174, 175] | |
| NOX4        | Human | aa 564–578 | pAb rabbit | n.s. | [176, 177] | |
| NOX4        | Human | n.s. | pAb rabbit | ~ 62 | [178, 179] | |
| NOX4        | Mouse | aa 88–103 | pAb rabbit | 55 and 60 | [180, 181] | |
| NOX4        | Mouse | aa 299–515 | pAb rabbit | 70–75 | [131, 182–188] | |
| NOX4        | Mouse | aa 307–578 | mAb mouse | ~ 65 | [36] | |
| NOX4        | Mouse | aa 553–572 | pAb rabbit | n.s. | [189] | |
| NOX4        | Rat | aa 81–95 and 566–578 | pAb rabbit | 62 | [190] | |

The table is not necessarily complete. Recommendations are based on self-assessed observations of the authors. No comment does not necessarily mean that the respective antibody is not recommended by the authors, as they have not tested all of them.

WB western blot, IF immunofluorescence, n.s. not specified, aa amino acid, pAb polyclonal antibody, mAb monoclonal antibody
validated regarding their overall and NOX isoform specificity. The necessity for confirming specificity was impressively underlined in a recent study [47], which showed that out of nine tested NOX4-directed siRNAs only six down-regulated murine NOX4 mRNA levels. Moreover, five of those six also down-regulated NOX1 mRNA levels. Another problem with investigating the role of NOX4 using siRNAs is the lack of specific antibodies against NOX4. Many if not all publications thus rely primarily on the down-regulation of NOX4 mRNA (see Table 1). These reports may need to be re-evaluated, as it was also recently shown that NOX4 is highly regulated at the post-transcriptional level, and therefore mRNA levels may not necessarily reflect protein levels and ROS formation [48, 49].

Antibodies against NOX

The lack of specific, freely available and validated antibodies against NOX1 and NOX4 represents one of the biggest roadblocks in the field. As described above, the validation of both siRNA-mediated down-regulation and genetic NOX1 and NOX4 KO models depends on the quality of the antibodies used for the characterization. Furthermore, as long as the tissue distribution of NOX1 and NOX4 remains unclear, it is very difficult to predict or estimate specific versus off-target effects of potential therapeutic interventions. Several groups and companies have attempted to generate polyclonal antibodies directed against different NOX1 and NOX4 peptides or recombinant proteins (Table 2). As these are polyclonal rabbit antibodies, the access and the amount were always limited. Also, several different protein sizes have been detected for NOX4 by different antibodies in the same tissues. This may be due to unspecificity of some antibodies, but also caused by the high sensitivity of the NOX4 protein to lysis conditions that may result in degradation and dephosphorylation [50]. So far, the polyclonal NOX4 antibodies by the Lambeth and Shah groups are the most frequently used. Of those antibodies which we have tested for isoform specificity, we recommend to use the NOX4 antibody from the Shah laboratory [51] and our NOX1 antibody [52]. In 2010, the successful generation of the first monoclonal mouse antibodies against human NOX4 was reported [50]; they were used to analyze the tissue distribution, subcellular localization, and structural features of NOX4 [17, 50]. Two of these antibodies (6B11 and 5F9) moderately block constitutive NOX4 activity in cell-free activity assays [50]. Another monoclonal antibody derived from rabbit is already commercially available, but no data have been published using this antibody in tissues and cells other than monocytes and macrophages [53]. These new antibodies may be promising and freely available tools for the validation of NOX1 and NOX4 as a therapeutic target. For NOX2, the commercially available antibody from Upstate Technologies (now Millipore, USA) is reliable in our hands.
several criteria: it should be active in cell-free conditions, an inhibitor [61]. An ideal NOX-inhibitor would have to fulfill calcium pump [60]. AEBSF is primarily a serine protease and eNOS [54, 58, 59], as well as cholinesterases and a inhibitor, also inhibiting, for example, xanthine oxidase and a compound have no intrinsic antioxidant activity, not inhibit other sources of ROS, and ideally be NOX isoform selective. To be applied as a tool for target validation, it should be effective in cells and tissues. For the development into a therapeutic drug, ADME must permit in vivo application and toxicity at an acceptable risk-to-benefit ratio. Recently, several NADPH oxidase-specific and even isoform-specific NOX inhibitors [62–66] have been published; we focus here on the first NADPH oxidase, but not isoform selective inhibitor, VAS2870 and its analogue VAS3947. For a detailed overview of the other interesting compounds, including the highly promising GKT136901, we refer to other publications [1, 64, 67, 68].

**Fig. 4** The role of NOX1, NOX2, and NOX4 in disease models. NO, generated by NO-synthases (NOS), activates soluble guanylate cyclase (sGC) by binding to its reduced (Fe^{2+}) heme moiety leading to the formation of cGMP from GTP. cGMP mediates protective effects, e.g. vasodilatation and anti-inflammation. This signaling pathway is most likely disturbed by NOX1-derived superoxide (O_2^{-}) as shown in Angiotensin II-induced hypertension and spontaneous hypertensive rats (SHR). Superoxide can either directly interact with NO to form peroxynitrite or oxidize the essential NOS cofactor tetrahydrobiopterin (BH_4) and thus uncouple NOS. Uncoupled NOS forms superoxide itself (not shown). Further, superoxide can oxidize the Fe^{2+} heme of sGC. Thereby, sGC becomes insensitive to NO. These mechanisms most likely account, at least in part, for the acute effects of increased NOX1 activity mediating endothelial dysfunction and the chronic effects that are discussed to cause hypertension. NOX2-derived superoxide is a major signaling molecule in innate immunity mediating host defense. NOX4 is unlikely to directly interfere with the NO/cGMP-signaling pathway as it releases hydrogen peroxide (H_2O_2) and not superoxide. However, in high concentrations, H_2O_2 causes acute cytotoxicity. This mechanism is suggested to be involved in NOX4-mediated effects after acute ischemic stroke, acute effects of pressure overload in heart, and bleomycin-induced cytotoxicity. The lower chronic activity of NOX4 seems to be involved in angiogenesis and wound healing, and thus rather protective.

**Pharmacological NOX inhibitors**

An important tool for the validation of potential therapeutic targets and proof of principle studies is the pharmacological inhibition by small chemical compounds. Several compounds have been used for many years, including apocynin, diphenylene iodonium (DPI), and 4-(2-aminoethyl)-benzensulfonylfluorid (AEBSF). However, it has become apparent that these inhibitors are not specific for NOX [1]. Apocynin cannot be used as selective NADPH oxidase inhibitor due to its direct antioxidant and several off-target effects [54–57]. DPI is a general flavoprotein oxidase inhibitor due to its direct antioxidant and several NADPH oxidase inhibition by VAS2870 and VAS3947 was observed in different cell-free assays including whole cell homogenates of A7r5 (mainly expressing NOX4, VAS3947 IC_50 of 13 μM) and CaCo-2 (mainly expressing NOX1, VAS3947 IC_50 of 12 μM) cell lines [59]. The ability to inhibit NOX2 can be concluded from experiments using either intact HL-60 cells (VAS2870 IC_50 of 1–2 μM) or isolated membranes of human neutrophils containing NADPH oxidase complexes formed from recombinant cytosolic subunits and NOX2 in the presence of SDS (VAS2870 IC_50 of 10.6 μM) [70, 71]. Furthermore, NADPH oxidase inhibition by VAS inhibitors could be detected in various native, i.e. non-overexpressing, cells expressing different NOX isoforms, including PMA-stimulated human granulocytes (expressing NOX2) [72] and DMSO-differentiated HL60 cells (mainly expressing NOX2) [59], several liver carcinoma cell lines [73], ox-LDL-treated human umbilical vein endothelial cells (HUVEC) [74], and PDGF-stimulated primary murine vascular smooth muscle cells [70]. In tissue samples, VAS2870 inhibits ROS release from aortas of aged spontaneous hypertensive rats (SHR) [59]. Also in endothelium-denuded rat tail arteries [75] and in hypoxic mouse brain...
slices [33], a significant decrease in ROS production was observed after VAS2870 treatment. In a mouse brain ischemia reperfusion model, NADPH oxidase activity was inhibited by in vivo treatment with VAS2870 [33], and in a zebrafish model of wound healing, DUOX was inhibited by VAS2870 [76]. In summary, VAS2870 is a well-validated NADPH oxidase inhibitor, as it shows no intrinsic antioxidant activity, does not inhibit other flavoproteins, inhibits NADPH oxidase-mediated ROS production in cell free systems, cells, tissues and in vivo, but it is not NOX isoform-specific. Very recently thioalkylation of cysteine residues of the ryanodine receptor Ca$^{2+}$ channel (RyR1) was discovered as a potential off-target effect of VAS2870 in sarcoplasmic reticulum vesicles isolated in glutathione (GSH) free buffer [193]. The authors also show binding of VAS2870 to low concentrations of GSH in vitro (10 μM). It will be interesting to know to which extent thioalkylation contributes in vivo to the mechanism of action of VAS2870 in the presence of physiological (mM) concentrations of GSH. However, for further development of the compound into a drug more extended off-target effects, ADME and safety data are required, including acute and chronic toxicity determination. So far, it has only been shown that VAS2870 does not inhibit ligand-induced platelet-derived-growth factor receptor (PDGFR)-tyrosine phosphorylation or PDGF-dependent phosphorylation of Erk1/2 or Akt [70].

Mechanism of action

In a cell-free system (membranes plus cytosol) VAS2870 only inhibited NOX2 activity when added prior to stimulation of the active complex formation between NOX2 and its cytosolic partners [71], whereas it showed no effect on NOX2 activity when added after stimulation of the complex formation with SDS (Fig. 3). This suggests that VAS2870 inhibits NADPH oxidase complex formation and can interfere with the association of NOX and its binding proteins. Surprisingly, the activities of NOX4 and NOX5, that are believed to be independent of cytosolic binding proteins, were also inhibited by VAS2870 when tested in native, mainly NOX4-expressing, A7r5 cells and NOX4 or NOX5 overexpressing HEK-293 cells, respectively (Fig. 3). Also, in vivo data suggest that VAS2870 does inhibit NOX4 in native systems: in a mouse ischemic stroke model, we observed the same protective effect of VAS2870 in the wild-type as by deletion of NOX4. VAS2870 exerted no additional protective effect in NOX4 KO mice [33]. Additionally, in endothelial cells from wild-type mice, pharmacological inhibition with VAS2870 or siRNA against NOX4 inhibited laminar shear stress-induced p38 MAPK activation mediated by hydrogen peroxide [77], and the effect was the same in endothelial cells from NOX4 KO mice (Santiago Lamas, personal communication). Recent data suggest an intramolecular interaction between unique motifs in C-terminus and cytosolic B-loop of NOX4 that forms a tertiary structure and activates H$_2$O$_2$ production [78, 79]. An intramolecular conformational change may also mediate the calcium-induced activation of NOX5 [31]. Thus, for all NOX isoforms, it is possible that inhibition of inter- or intramolecular conformational changes is a common mechanism of action of VAS2870. Thioalkylation of critical cysteine residues of NOX enzymes by VAS2870 was recently, e.g. the cytosolic B-loop, suggested [193], but the molecular details and binding sites of this remain to be elucidated.

Applying the tools: validated targets and possible indications

It is still early days in NOX research, and certainly with respect to translation. Nevertheless, what can already be said about validated roles of NOX and NADPH oxidase in disease? And which of these roles may be translated into therapeutic indications? Different NOX subunits have been suggested to be implicated in cancer, hypertension, lung fibrosis, stroke, heart failure, diabetes, and neurodegenerative diseases [18]. Several principal ways may be differentiated by which an excess of ROS leads to pathology: spatially confined levels of ROS (e.g., in caveolae) that interfere with nitric oxide’s (NO) vasoprotective signaling, and high levels (local or systemic) that act, at least in part, independently of NO and are directly cytotoxic, cause apoptosis (Fig. 4), or disturb redox-sensitive signaling pathways.

Roles of NOX1, NOX2, and NOX4

NOX2 appears to be relevant in almost every disease model tested. This may be connected to the role of NOX2 in the innate immune response [80], including to fungal infections [81, 82] and adaptive immune response at the level of both T cells and antigen-presenting cells [83, 84]. Thus, in an animal model involving a significant inflammatory response, NOX2 inhibition may lead to an improvement. Whether this can be exploited in light of the essential immune functions of NOX2 is an important question. Importantly, even a small residual NOX2 activity in X-linked chronic granulomatous disease (CGD) is sufficient for a functional innate immune system [85]. However, it is unknown whether a partial pharmacological inhibition of NOX2 will sufficiently suppress NOX2’s non-CGD disease-related activity. In addition, chronic NOX2 inhibition might lead to paradoxical autoimmune responses [86]. Rather, one may want to optimize any NOX inhibition approach by leaving NOX2 unaffected.

With respect to low and spatially confined ROS overproduction, NOX1 is a good candidate to migrate into
caveolae and there cause eNOS uncoupling and endothelial
dysfunction, which is often associated with increased blood
pressure and enhanced platelet aggregation. Moreover, it
may be an early step in the development of atherosclerosis.
Indeed, basal blood pressure [39], angiotensin-induced
hypertension [39, 40], and endothelium-dependent relaxation
in spontaneously hypertensive rats [87] depends—to some degree—on NOX1. However, whether such chronic
disease indications would ever become realistic for NOX
inhibition is highly questionable unless sophisticated
patient stratification biomarkers would become available.
Phosphorylation of vasodilator-stimulated phosphoprotein
(P-VASP) could become such a marker [88].

With respect to higher levels of ROS that act, at least in part,
independently of NO and are directly cytotoxic or cause apoptosis, NOX4 is well validated. NOX4 is induced
in ischemic stroke, in pressure overload of the heart, and in
a bleomycin model of lung epithelial toxicity resulting in
lung fibrosis. Whilst the interpretation of the stroke data obtained with NOX4 KO mice is straightforward and was
recently confirmed in a tgNOX4 model of brain ischemia
showing larger infarct sizes [194], the pressure overload
and lung data are less so. In pressure overload, two models
have been applied, proximal aortic or thoracic aortic constriction (TAC), and abdominal aortic banding. Both
models differ in the time course by which they affect the heart. The latter, less acute model allows for angiogenesis
to occur. NOX4 appears to play a double role by contributing to the cardiomyocyte damage (particularly in the
acute TAC model [32]) and by facilitating subacute angiogenesis and promoting cardiac function (only observable
in the subacute abdominal aortic banding). This may explain why opposing phenotypes were observed in both
NOX4 KO mouse models and different disease models. In
particular, the TAC model was tested in a cardiomyocyte-specific KO and therefore leaves vascular cell-dependent
angiogenesis by definition unaffected. Thus, NOX4 might both acutely damage the cardiomyocyte and subacutely
protect the heart by promoting angiogenesis. NOX4 also
promotes angiogenesis in vitro as shown using HUVEC
[89, 90] and ovarian cancer cells [91]. Whether these
effects may be exploited by defining an optimal time
window for NOX4 inhibition in situations of acute heart
failure or by interfering with tumor angiogenesis remains
to be seen, and it needs to be tested by TAC or cancer
models in a global KO animal and by applying NOX
inhibitors. The situation in the lung is similarly compli-
cated. Here, a role of NOX4 in the pathogenesis of hypoxic
pulmonary hypertension was suggested [92], but not con-
firmed in NOX4 KO mice [33]. Recent data showed that
NOX4 deficiency mediated either by NOX4 siRNA [93],
NOX4 inhibition, or NOX4 deletion [35] prevents lung
fibrosis. However, this observation may be model-
dependent as no protection from lung fibrosis was observed
in another NOX4 KO mouse using the same model
(Weissmann N. and Schmidt H.H.H.W., unpublished
observation). Bleomycin induces apoptosis and inflamma-
tion in mouse lung epithelial cells [35]. Thus, NOX4 may
be relevant in the bleomycin model, but this model may not
reflect the wide spectrum of human lung fibrosis (idiop-
atric, radiation, silicosis, systemic lupus erythematosus,
dermatomyositis, scleroderma, rheumatoid arthritis,
pneumoconiosis, acute respiratory distress syndrome,
chronic heart failure, drug-induced). Thus, a model-inde-
pendent role of NOX4 in lung fibrosis needs to be tested in
different models of the disease. Even then, the clinical chal-
lenge of a life-long therapy with a NOX4 inhibitor would
remain. Importantly, all published NOX4 KO models lack a
basal phenotype. This is an important observation for the
caracterization of NOX4 as a therapeutic target, as it indi-
cates that NOX4 inhibition would probably not cause severe
complications. The situation may be different when co-mor-
bidities occur and protective roles of NOX4 may well cause
da side effects. From the current state of knowledge, such
potential side effects of sub-chronic and chronic NOX4
inhibition could arise from decreased angiogenesis.

In conclusion, according to the current knowledge, acute
ischemic stroke appears to be one of the most promising
and safest targets for NOX inhibition. It evades the risk of
chronic therapy and the rather double-edged role of NOX4
in heart failure and angiogenesis. Nevertheless, specific,
isoform-selective NOX inhibitors and reliable, freely
available antibodies will be key in elucidating the full
therapeutic potential of NOX in species other than mouse
and in different disease models.

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vasopharm GmbH (Würzburg, Germany), which pharmaceutically
develops NADPH oxidase inhibitors. H.H.H.W.S. and K.W. are
inventors of a patent on VAS2870 and VAS3947, which is owned by
vasopharm GmbH (Würzburg, Germany). K.W. is a former, P.S. is
currently an employee of vasopharm GmbH (Würzburg, Germany).

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