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MULTI-AUTHOR REVIEW

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.

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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 [1] 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 H_2O_2 [17]. All NOX isoforms have been reported to bind to one or more membrane and/or cytosolic proteins. $p22^{phox}$ 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, $p47^{phox}$ and $p67^{phox}$, and also the cytosolic protein, $p40^{phox}$ [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

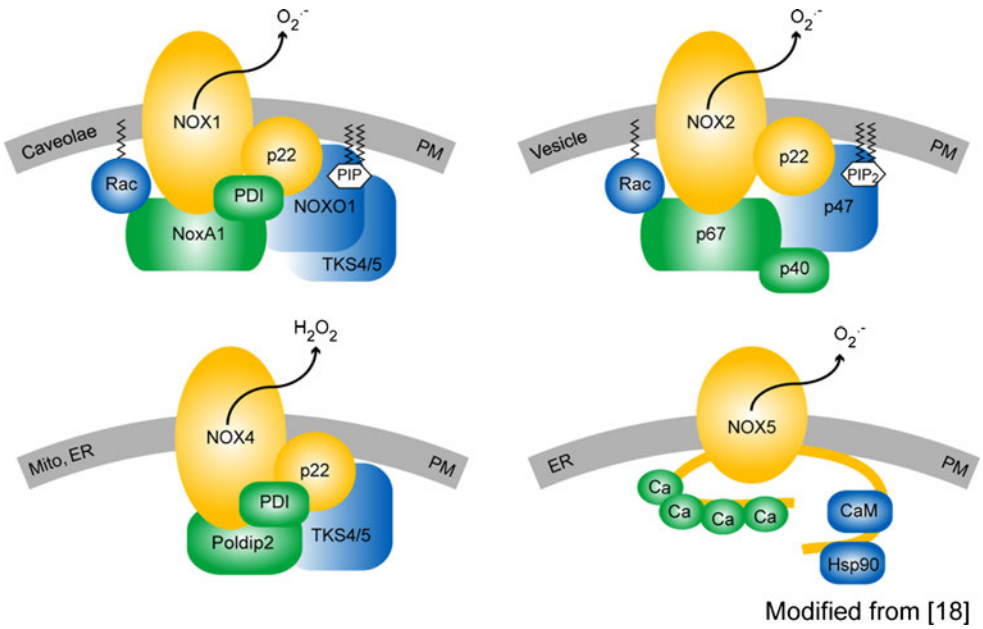

Fig. 1 The vascular NOX isoform-based NADPH oxidase complexes. Cell or subcellular compartment membranes are shown in *gray*, core proteins in *yellow*, activator binding proteins in *green* and organizer binding proteins in *blue*. All the NOX isoforms shown are membrane proteins and are localized in 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 $p22^{phox}$, but only NOX1 and NOX2 are regulated by the small GTPase Rac. For its activation, the NOX1 enzyme complex requires the assembly of NOX organiser 1 (NOXO1) and NOX activator 1 (NOXA1), but also forms complexes with $p47^{phox}$ and $p67^{phox}$ (not shown). The NOX2 enzyme complex requires binding of $p47^{phox}$, $p67^{phox}$, and optionally $p40^{phox}$ 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 $p47^{phox}$ 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]

Table 1 NOX4 siRNA approaches: this table provides a selection of published siRNAs used for downregulation of NOX4

NOX isoform	Species	Sequence	Degree of NOX4 down-regulation (% of ctr.)	Ref./source	Comment
NOX4	Bovine	5′-AAGACCTGGCCAGTATATATTAT-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′-GUUCUUAACCUCAAGUGCATT-3′ (sense); 5′-UGCAGUUGAGGUUUAAGAACTT-3′ (antisense)	n.q. (protein, mRNA)	[97]	Base error according to database sequence
NOX4	Human	5′-UUAUUGCAUAUGUAGAGGCUGUGAU-3′ (sense); 5′AUCACAGCCUCUACAUAUGCAAUAA-3′ (antisense)	n.q. (mRNA)	[98]	
NOX4	Human	5′-GUCAACAUCCAGCUGUACCdtdt-3′ (sense), 5′-GGUACAGCUGGAUGUUGACdtdt-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) ACUAUGAUAUCUUCUGGUA; (2) GAAAUUAUCCCAAGCUGUA; (3) GGGCUAGGAUUGUGUCUAA; (4) GAUCACAGCCUCUACAUAU	n.q. (mRNA)	Dharmacon [101]	
NOX4	Human	Targets exon2	40 % (mRNA and protein)	Ambion [102, 48]	ID #118807
NOX4	Human	5′-XCACCACCACCACCACCATT-3′; 5′-AAUGGUGGU GGUGGUGGUGTT-3′	n.s.	[103]	
NOX4	Human	n.s.	n.q. (protein)	Qiagen [104]	Hs_NOX4_1 and Hs_NOX4_2 predesigned
NOX4	Human	5′-CAGAACATTCCATATTAC-3 & 5′-ACTTTGTTGAACTGAATG-3′	n.q. (mRNA)	[105]	
NOX4	Human	Mixture of: (1) 5′-AAAGCAGGACAU UCAUGGA GAGCCA-3′ (sense); 5′-UGGCUCUCCAUGAAUGUCC UGGCUUU-3′ (antisense); (2) 5′-GCAUCUGUUCUUAACCUCA-3′ (sense); 5′-UGAGGUUAAGAACAGAUGC-3′ (antisense); (3) 5′-CCAGGAGAUUGUUGGAUAA-3′ (sense); 5′-UUAUCCAACAAUCUCCUGG-3′ (antisense); (4) 5′-CAGUGAAGACUUUGUUGAACUGAAU-3′ (sense); 5′-AUUCAGUUCAACAAAGUCUUCACUG-3′ (antisense)	~40 % (mRNA)	[106]	Sequences not present in NOX1, NOX2, NOX3, and NOX5
NOX4	Human	5′-AGACCUGGCCAGUAUAUUA-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) ~75 % (protein)	[109]	
NOX4	Human	5′-CGAGAUGAGGAUCCUAGAAdTdT-3′ (sense); 5′-UUCUAGGAUCCUCAUCUCGdTdT-3′ (antisense)	~25 % (mRNA)	[90]	

Table 1 continued

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′-AAACCGGCAGGAGUUUACCCAG-3′	~45 % (protein) n.q. mRNA	[110, 111]	
NOX4	Human	5′-GTCAACATCCAGCTGTACCdTdT-3′	n.q. (mRNA)	[112, 113]	
NOX4	Human	(1) 5′-GATCCGCAGAACATTCCATATTACT ICAAGAGAGTAATA TGGAATGTTCTGCTTTTTTGGAAA-3′ (2) 5′-GATTCCGACTTTGTTGAACTGAATGTTCAAG AGACATTC AGTTCAACAAAGTCTTTTTTGGAAA-3′	n.q. (mRNA and protein)	[114]	
NOX4	Human	(1) 5′-GAAUUACAGUGAAGACUUU-3′ (sense); 5′-AAAGUCUUCACUGUAAUUC-3′ (antisense); (2) 5′-CAGGAGGGCUGCUGAAGUA-3′ (sense); 5′-UACUUCAGCAGCCCUCCUG-3′ (antisense); (3) 5′-GGGCUAGGAUUGUGUCUAA-3′ (sense);5′-UUAGACACAAUCCUAGCCC-3′ (antisense); (4) 5′-GAUCACAGCCUCUACAUAU-3′ (sense); 5′-AUAUGUAGAGGCUGUGAUC-3′ (antisense)	n.q. (protein)	[93]	(1) and (2) not efficient, (4) most efficient
NOX4	Human, rat	5′-ACUGAGGUACAGCUGGAUGUU-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′-GGCCAACGAAGGGGUUAAACACCUC-3′	n.q. (mRNA)	[118, 119]	
NOX4	Mouse	5′-GGAUAAAAGCAAGACUCUACACAUC-3′	(mRNA)	[119]	
NOX4	Mouse	Mix of 3 siRNAs: 5′-CCAUUUGCAUCGAUACUAA-3′; 5′-CCAAGACUCUUCAUAGUUU-3′; 5′-CAAGACCUCUCUCCUUUGA-3′	40 % (mRNA)	Santa Cruz [120]	
NOX4	Mouse	Target sequence: 5′-CAGGAATAAATTAAAGCTTTA-3′	n.s.	[121]	
NOX4	Mouse	28-kDa NOX4 (5′-AATGTTGGGCTGTCCTACTGA-3′ (sense) UGUUGGGCUGUCCUACUGAdTdT (antisense), UCAGUAGGACAGCCCAACAdTdT and full-length 65 kDa and 28 kDa (5′-AACGAAGGGGTTAAACACCTC-3′ and 5′-AAAAGCAAGACTCTACACATC-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′-AACGAAGGGGTTAAACACCTC-3′, (2) 5′-AAAAGCAAGACTCTACACATC-3′	n.q. (protein)	[126]	
NOX4	Mouse	5′-GGUUACAGCUUCUACCUAC-3′ (sense); 5′-GUAGGUAGAAGCUGUAACC-3′ (antisense)	n.q. (protein and mRNA)	Dharmacon [93]	In vivo treatment
NOX4	Pig	n.s.	50-60% (protein)	Dharmacon [127]	Tested vs. NOX2

Table 1 continued

NOX isoform	Species	Sequence	Degree of NOX4 down-regulation (% of ctr.)	Ref./source	Comment
NOX4	Rat	siRNA against Nox4 5′-AACGAAGGGTTAAACACCTC-3′	~40 % (mRNA), n.q. (protein)	[128]	Tested vs. NOX1
NOX4	Rat	n.s.	~50 % (protein and mRNA)	Dharmacon [129]	
NOX4	Rat	5′-GUAGGAGACUGGACAGAAA-3′ (sense)	n.d.	[130]	
NOX4	Rat	(1) 5′-GUUAGUCUGUGUGUGGCUGtt-3′, (2) GAUUUGCCUGGAAGAACCCtt-3′	n.d.	[131]	

The table is not necessarily complete. Species specificity is shown as published and/or as tested by the authors, but may be limited to the stated species. Recommendations are based on self-assessed observations of the authors. No recommendation does not necessarily mean that the respective siRNA is not recommended, as the authors did not test all siRNAs

n.s. not specified, *n.q.* not quantified, *ctr.* control

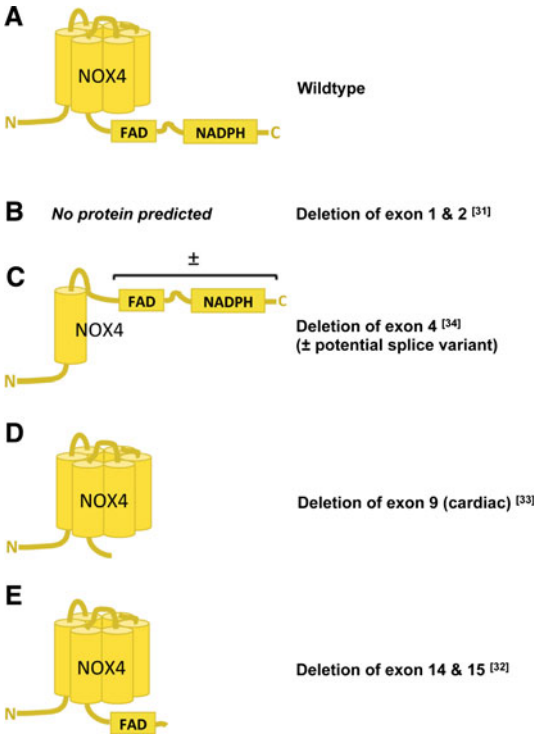

Fig. 2 Published NOX4 knock-out (KO) mouse models. **a** Wild-type NOX4 has six transmembrane helices and cytosolic binding domains for FAD and NADPH at the C-terminus. **b** Deletion of exons 1 and 2 should delete the complete NOX4 protein [32]. **c** Deletion of exon 4 only leaves the first transmembrane domain of NOX4. However, hypothetically, this may also result in the formation of a splice variant that contains both FAD and NADPH binding domains and thus has remaining ROS-forming activity [43]. **d** Another knock-out was generated by conditionally deleting exon 9 of NOX4 in cardiomyocytes, thereby deleting the FAD binding domain, likely leaving a non-functional enzyme [34]. **e** The fourth published NOX4 KO mouse was generated by deleting exons 14 and 15 that refer to the NADPH binding domain. This likely results in the expression of a non-functional enzyme [33]

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 NOX 5 [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 $p22^{phox}$ 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 $p22^{phox}$ 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.

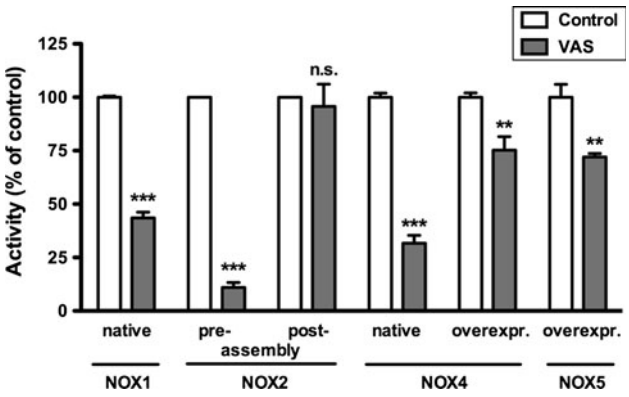

Fig. 3 VAS2870 inhibits assembly of NADPH oxidases. *NOX1* whole cell homogenates of CaCo-2 cells (native) were prepared and ROS measured as described in presence or absence of VAS3947 (30 μM) [59]. *Columns* represent means ± SEM of $n = 3$ experiments normalized to untreated controls. *NOX2* membranes of human neutrophils, Rac-2-enriched cytosol fraction as well as recombinant $p47^{phox}$ and $p67^{phox}$ were treated with SDS to induce assembly of these subunits as described [71]. VAS2870 (55 μM) or a solvent control were added before (pre-) or after (post-) assembly of NOX2 with its subunits, and NADPH oxidase activity was measured using the cytochome c reduction assay as described [71]. *Columns* represent means ± SEM of $n = 3$ experiments normalized to untreated controls. *NOX4* whole-cell homogenates of A7r5 cells (native), mainly expressing NOX4 compared to other NOX isoforms, were prepared and ROS measured as described in presence or absence of VAS3947 (30 μM) [59]. *Columns* represent means ± SEM of $n \geq 5$ experiments normalized to solvent treated controls. Untransfected HEK293 cells (not shown) or HEK293 cells stably transfected with human NOX4 (overexpr.) were treated with VAS2870 or solvent control, and H_2O_2 release was measured using Amplex Red. Briefly, Amplex Red (20 μM) and horseradish peroxidase (100 mU/ml) in a phosphate buffer containing VAS2870 (10 μM) or equal volumes of solvent as control were incubated for 10 min at 37 °C in the dark in a 96-well plate. Then, 10^5 native or human NOX4 overexpressing HEK293 cells were added to the wells, and fluorescence was recorded for 60 min in a Wallac Victor V (Perkin Elmer Life Sciences, Waltham, MA, USA) or Spectramax M2 (Molecular Devices, Sunnyvale, CA, USA) plate reader using 540/590 nm excitation/emission wavelength filters. *Columns* represent means ± SEM of the AUC of time-dependent fluorescence curves in quadruplicates of $n = 4$ experiments normalized to non-VAS treated HEK293-NOX4 cells. *NOX5* L012 (100 μM) was used to measure NOX5 activity in HEK293 cells stably transfected with human NOX5 beta. VAS2870 (10 μM) or a solvent control was added to the cells in a 96-well plate, and basal chemiluminescence was recorded in a Victor V plate reader with 10 readings per well. Then, NOX5 was stimulated with phorbol myristate acetate (PMA, 1 μM) and the calcium ionophore ionomycin (1 μM), or HBSS as control was added (not shown), and chemiluminescence was measured for 20 readings per well for 60 min. *Columns* represent means ± SEM of AUC of time-dependent chemiluminescence in quadruplicates normalized to VAS2870 solvent control. (***$p < 0.001$, **$p < 0.01$ are significantly different from control values; *n.s.* $p > 0.05$ not significantly different from control values; 1-way ANOVA calculated with GraphPad Prism5 for each individual experiment)

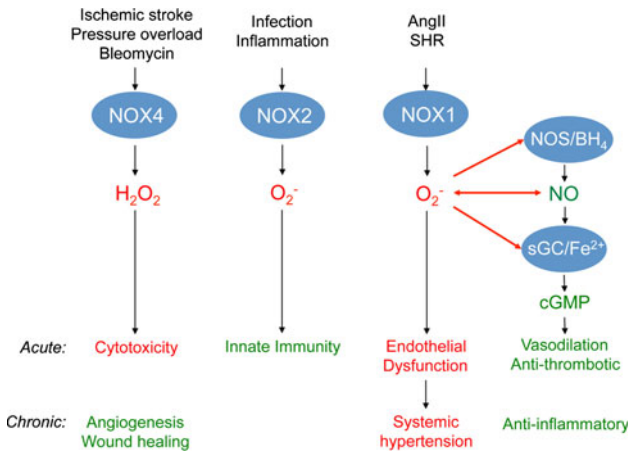

Fig. 4 The role of NOX1, NOX2, and NOX4 in disease models. NO, generated by NO-synthases (NOS), activates soluble guanylate cyclase (sGC) by binding to its reduced (Fe^{2+}) heme moiety leading to the formation of cGMP from GTP. cGMP mediates protective effects, e.g. vasodilation and anti-inflammation. 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 tetrahydrobioapterin (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 inhibitor, also inhibiting, for example, xanthine oxidase and eNOS [54, 58, 59], as well as cholinesterases and a calcium pump [60]. AEBSF is primarily a serine protease inhibitor [61]. An ideal NOX-inhibitor would have to fulfil several criteria: it should be active in cell-free conditions, 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].

The NADPH oxidase inhibitors VAS2870 and VAS3947

The first published inhibitors that resulted from a systematic screening effort for selective NADPH oxidase inhibitors were the triazolo pyrimidines, represented by the commercially available VAS2870 and its derivatives, such as VAS3947 [69]. VAS3947 shows an improved solubility but does not differ in its inhibition profile (unpublished data). In contrast to formerly used NADPH oxidase inhibitors, the VAS compounds do not show intrinsic antioxidant activity nor do they inhibit other flavoproteins such as eNOS and xanthine oxidase [59].

Validation of the VAS compounds

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_2O_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 any 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 complicated. Here, a role of NOX4 in the pathogenesis of hypoxic pulmonary hypertension was suggested [92], but not confirmed 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 inflammation 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 (idiopathic, radiation, silicosis, systemic lupus erythematosus, dermatomyositis, sclerodermia, rheumatoid arthritis, pneumoconiosis, acute respiratory distress syndrome, chronic heart failure, drug-induced). Thus, a model-independent role of NOX4 in lung fibrosis needs to be tested in different models of the disease. Even then, the clinical challenge 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 characterization of NOX4 as a therapeutic target, as it indicates that NOX4 inhibition would probably not cause severe complications. The situation may be different when co-morbidities occur and protective roles of NOX4 may well cause 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.

Acknowledgments H.H.H.W.S. declares that he holds shares in 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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