Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets

Grant R. Drummond[*], **Stavros Selemidis**[*], **Kathy K. Griendling**[‡], and **Christopher G. Sobey**[*]

[*]Vascular Biology & Immunopharmacology Group, Department of Pharmacology, Monash University, Victoria 3800, Australia.

[‡]Emory University, Division of Cardiology, Atlanta, Georgia 30322, USA.

Abstract

NADPH oxidases are a family of enzymes that generate reactive oxygen species (ROS). The NOX1 (NADPH oxidase 1) and NOX2 oxidases are the major sources of ROS in the artery wall in conditions such as hypertension, hypercholesterolaemia, diabetes and ageing, and so they are important contributors to the oxidative stress, endothelial dysfunction and vascular inflammation that underlies arterial remodelling and atherogenesis. In this Review, we advance the concept that compared to the use of conventional antioxidants, inhibiting NOX1 and NOX2 oxidases is a superior approach for combating oxidative stress. We briefly describe some common and emerging putative NADPH oxidase inhibitors. In addition, we highlight the crucial role of the NADPH oxidase regulatory subunit, p47phox, in the activity of vascular NOX1 and NOX2 oxidases, and suggest how a better understanding of its specific molecular interactions may enable the development of novel isoform-selective drugs to prevent or treat cardiovascular diseases.

The primary catalytic function of the NADPH oxidase family of enzymes is the generation of reactive oxygen species (ROS). This property sets them apart from all other ROS-generating enzymes that produce radical species, either as a by-product of their normal catalytic activity or as a result of aberrant functioning in disease. Members of the NADPH oxidase family are expressed in most — if not all — mammalian cell types, in which they catalyse the reduction of molecular oxygen to generate superoxide and/or hydrogen peroxide in various intracellular and extracellular compartments. The ROS generated by NADPH oxidases have crucial roles in various physiological processes, including innate immunity, modulation of redox-dependent signalling cascades, and as cofactors in the production of hormones.

For several decades, it has been recognized that the rare condition known as chronic granulomatous disease (CGD; see BOX 1)[1] is caused by an underactive NADPH oxidase system, in which the capacity of phagocytic leukocytes to generate a microbicidal burst of ROS is impaired, leaving the individual susceptible to severe, life-threatening infections by opportunistic microbes. By contrast, it has only recently emerged that excessive ROS

production by an overactive NADPH oxidase system, both in phagocytic and non-phagocytic cell types of the artery wall, may set in motion a vicious cycle of radical and non-radical oxidant generation in various cellular compartments, which disrupts redox circuits that are normally controlled by thiol-dependent antioxidant defences[2,3]. This induces a state of oxidative stress, which is necessary for the initiation and progression of vascular disease that may ultimately lead to heart attacks and strokes.

The knowledge that ROS are involved in the pathogenesis of vascular disease should facilitate the development of therapies that directly target ROS production to prevent cardiovascular events. However, most current cardiovascular therapies are still merely aimed at either reducing a patient's risk profile (for example, by lowering blood pressure or reducing cholesterol levels) or alleviating the symptoms of vascular disease (for example, by reducing the cardiac workload to prevent angina attacks). Hence, existing approaches do not yet adequately address the ongoing oxidative processes in the diseased vessel wall, and thus it may be speculated that with current cardiovascular therapies patients remain at an increased risk of a cardiovascular event. It has therefore been disappointing to find that antioxidant drugs (for example, vitamins C and E, and β-carotene) do not appear to have the clinical efficacy to prevent cardiovascular events[4], even though these drugs can, high concentrations, chemically remove ROS in *in vitro* systems and in experimental animal models of cardiovascular disease. However, given that conventional antioxidants display poor reactivity with many endogenous ROS, in retrospect the failure of these clinical trials perhaps not so surprising. Therefore, targeting ROS production by blocking the activity of culprit enzymes — such as NADPH oxidases — is likely to be a far superior approach for combating oxidative stress and preventing the progression of vascular disease than previous attempts aimed at scavenging ROS.

This Review provides an overview of the mechanisms by which ROS can cause vascular disease and the probable reasons why previous attempts to eliminate these species with antioxidants failed. We summarize the evidence that NADPH oxidases are key generators of ROS in the blood vessel wall and other tissues during cardiovascular disease progression. We also discuss why targeting these enzymes in an isoform-selective manner (by exploiting specific molecular features of the different isoforms) is likely to offer therapeutic advantages over conventional antioxidants while having minimal impact on immune cell function by professional phagocytes or on ROS-dependent processes that are essential for normal cell function.

Oxidative stress

A link between risk factors and vascular disease

Vascular disease is a general term that encompasses several pathological states of the arterial wall that may lead to obstructed blood flow and thereby give rise to acute cardiovascular events. Such pathological states include arterial remodelling, atherosclerosis, thrombosis and restenosis. Cardiovascular risk factors — such as hypertension, hypercholesterolaemia, smoking, obesity, diabetes and ageing — almost invariably precede the onset of vascular disease, which is indicative of a causal link. Oxidative stress is associated with all known cardiovascular risk factors, and a large body of evidence indicates that it is implicated in many of the processes that are involved in the formation of atherosclerotic plaques[5,6]. These observations highlight that oxidative stress is a link between cardiovascular risk factors and vascular disease, and thus a compelling target for disease prevention or therapy.

Oxidative stress is defined as an excess in the levels of of oxidants over antioxidants within a biological system, and the direct consequence of this is a shift in the redox state of the biological compartment (for example, the nucleus, mitochondria, cytosol or extracellular

space) towards one that is more oxidizing[2,7]. ROS — which include radical species such as superoxide anions and hydroxyl radicals, and non-radical oxidants such as hydrogen peroxide — are particularly important effectors of cellular redox status. It is the overproduction of these species that is considered to be a major cause of oxidative stress in the arterial walls of individuals with cardiovascular risk factors, and in organs such as the heart, brain and kidneys following ischaemia–reperfusion injury[7,8].

Oxidative stress not only causes direct and irreversible oxidative damage to macromolecules but also disrupts key redox-dependent signalling processes in the arterial wall[9]. Perhaps the best characterized mechanism by which oxidative stress can promote vascular disease is via disruption of the vasoprotective nitric oxide (NO) signalling pathway[10]. It has been known for many years that superoxide anions can chemically react with and inactivate NO. This nullifies the anti-inflammatory and vasodilator functions of NO, and promotes the formation of peroxynitrite[11,12]. Peroxynitrite is a powerful oxidant in its own right, and may thus contribute to oxidative stress through oxidation of small-molecule antioxidants such as glutathione, cysteine and tetrahydro—biopterin[13]. Reduced bioavailability of tetrahydrobiopterin results in uncoupling of endothelial nitric oxide synthase (eNOS), which causes it to change from being an NO-producing, vasoprotective enzyme to being a superoxide-producing, oxidative stress-causing enzyme[14,15]. Peroxynitrite can also oxidatively inactivate antioxidant enzymes, such as glutathione reductase, glutaredoxin and superoxide dismutase[13], as well as dimethylarginine dimethylaminohydrolase, which metabolizes the endogenous inhibitor of eNOS — asymmetric dimethylarginine[16]. Finally, oxidative stress can render the receptor for NO dysfunctional, as the NO-binding haem group of soluble guanylyl cyclase, which normally exists in the Fe^{2+} state, is highly susceptible to oxidation to the Fe^{3+} state, which is associated with a markedly reduced affinity of the enzyme for NO[17].

In addition to inactivation of NO, ROS may directly promote vascular inflammation and remodelling. Indeed, many adverse effects of ROS on the arterial wall are attributable to the oxidation of key signalling proteins, including kinases and phosphatases, and activation of the pro-inflammatory redox-dependent transcription factor NF-κB[18]; this leads to the expression of adhesion molecules on the endothelium[19], and the proliferation and migration of vascular smooth muscle cells (VSMCs)[20]. ROS may also oxidize matrix metalloproteinases, leading to their activation and the subsequent promotion of vascular remodelling[21]. Moreover, it was recently suggested that ROS initiate the assembly of multiprotein signalling complexes known as inflammasomes, which activate caspase 1, and thereby lead to the processing and secretion of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18 (REF. 22).

Why haven't antioxidants worked?—Previous strategies to alleviate oxidative stress in patients with cardiovascular disease aimed to bolster levels of antioxidants — especially vitamin E — within target tissues by increasing antioxidant intake. However, despite evidence from cohort studies of an inverse relationship between dietary or supplemental vitamin E intake and the development of cardiovascular disease[23], several randomized clinical intervention trials have failed to show any net benefit, either by arresting progression of atherosclerosis or by preventing the incidence of further cardiovascular events[24–26].

However, owing to limitations in the design of the clinical trials and the inability of most conventional antioxidants to neutralize all ROS and reverse oxidative stress *in vivo*, these observations should not be regarded as evidence against a role for oxidative stress in cardiovascular disease. First, most of these trials were performed in high-risk patients in which vascular disease was presumably quite advanced, and so it is plausible that when

antioxidant therapy was commenced, it was too late for it to have a substantial protective effect.

Second, the doses of vitamin E used varied greatly between trials (that is, 30–800 mg) and in no trial was it determined whether the treatment actually affected the antioxidant concentration at the target site of the disease (that is, the blood vessel wall). Indeed, this issue of antioxidant concentration within the vessel wall is extremely important considering the exceptionally high rate constants of the reactions between ROS and crucial endogenous molecules such as NO and certain amino acids and nucleic acids. Reactions between these molecules and ROS must therefore be outcompeted by the therapeutic antioxidant for it to effectively remove ROS from the blood vessel wall and positively affect vascular health. For example, the reaction between superoxide and NO occurs at a rate of approximately $1.9 \times 10^{10} \, M^{-1} \, s^{-1}$ (REF. 27), which is six orders of magnitude faster than the reaction between superoxide and vitamin E[28]. Thus, for vitamin E to prevent this reaction from occurring in a given cellular compartment, its concentration must exceed that of NO by one million-fold. Given that concentrations of NO within the vessel wall are estimated to be in the low nanomolar range[29], this would require a vitamin E concentration in the millimolar range, which is unlikely to be achievable by even the highest levels of vitamin E intake.

Third, even if sufficient concentrations of vitamin E were achieved in appropriate cellular compartments within the blood vessel wall, it is likely that vitamin E would be rapidly oxidized in such an oxidizing environment without any prospect of regeneration. Such a situation would not only lead to rapid depletion of the reduced (active) form of the antioxidant but may also cause accumulation of the oxidized form of the parent molecule, which in some instances (including for the vitamin E radical) may exert its own pro-oxidant and pro-atherogenic effects[30,31].

Finally, it is noteworthy that although the reaction of certain ROS with antioxidants may lead to a reduction in the concentration of that particular ROS, the product of the reaction may simply be another type of ROS that does not react with the original antioxidant, and may not be further neutralized by an already overwhelmed endogenous antioxidant defence system[32].

Therefore, prevention of ROS formation by targeting the enzymes responsible for their generation could be a more effective strategy for ameliorating oxidative stress than scavenging these highly reactive molecules once they are formed. Such an approach requires knowledge of the key enzymes that are responsible for ROS production in vascular disease, including their cellular localization. The following section outlines the evidence for NADPH oxidases as important ROS-generating enzymes in vascular disease.

Isoforms of NADPH oxidase in vascular disease—Since their initial identification in endothelial cells and VSMCs[33,34], a large and rapidly expanding body of experimental evidence, including studies in knockout mice (TABLE 1), implicates NADPH oxidases in the constitutive cells of the artery wall, as well as in leukocytes and brain cells, as underlying causes of oxidative stress in various cardiovascular disease settings. NADPH oxidases are a family of multisubunit enzyme complexes that are unique in being the only enzymes that have been identified with the primary function of generating superoxide and/or hydrogen peroxide.

Seven isoforms of NADPH oxidase have been described in mammals. Each of these isoforms comprises a core catalytic subunit — the so-called NADPH oxidase (NOX) and dual oxidase (DUOX) subunits — and up to five regulatory subunits. These regulatory subunits have important roles in: the maturation and expression of the NOX and DUOX

subunits in biological membranes (p22phox, DUOX activator 1 (DUOXA1) and DUOXA2); in enzyme activation (p67phox and NOX activator 1 (NOXA1)); and in spatial organization of the various components of the enzyme complex (p47phox, NOX organizer 1 (NOXO1) and p40phox)[35]. Some NADPH oxidase isoforms also rely on a small GTPase (RAC1 or RAC2) for their activation. As illustrated in FIG. 1, each NADPH oxidase isoform may be defined by the nature of its core catalytic subunit (NOX1–NOX5, DUOX1 or DUOX2) as well as its suite of regulatory subunits.

Mechanism of action of NADPH oxidases

NADPH oxidases catalyse the transfer of electrons from the cytosol across biological membranes and into various intracellular and extracellular compartments. Depending on the biological membrane in which the NADPH oxidase is expressed, such cellular compartments can include the nucleus, endoplasmic reticulum, endosome, phagosome, mitochondria and extracellular space[20]. Cytosolic NADPH acts as the electron donor for all NADPH oxidase isoforms. When the enzymes are active, electrons are abstracted from NADPH and transferred along an electron transport chain contained within their catalytic subunit. This consists of a single FAD molecule bound to the extended carboxy-terminal tail, and two prosthetic haem groups attached to histidine residues within the transmembrane region of the protein. Oxygen invariably acts as the final electron acceptor. For the NOX1, NOX2 (formerly called gp91phox) and NOX5 oxidases, electron transfer to molecular oxygen results in the release of superoxide from the enzyme. Because superoxide is an anionic molecule, its generation within specific cellular compartments needs to be charge-compensated and, for NOX1 at least, this appears to be achieved by the actions of the chloride–proton antiporter ClC3 (REF. 36). Surprisingly, despite the overall structural similarities among the seven NADPH oxidase family members, NOX4, DUOX1 and DUOX2 oxidases do not appear to generate superoxide as their primary ROS. Instead, the activity of these enzymes results in the direct release of hydrogen peroxide[37,38]. The biochemistries underlying the direct generation of hydrogen peroxide by NOX4, DUOX1 and DUOX2 oxidases are yet to be fully characterized. However, it has been suggested that the dissociation of superoxide from the catalytic subunit of these enzymes may be delayed, possibly owing to hindrance by the third extracytosolic loop, thus allowing time for the generation of a second molecule of superoxide and its subsequent dismutation prior to its release from the enzyme[38,39].

NADPH oxidases in the constitutive cell types of the artery wall

NADPH oxidases are an important source of ROS in blood vessels, and multiple NOX oxidase isoforms are constitutively expressed in each of the predominant cell types of the vascular wall[40]. Endothelial cells express NOX1 oxidase, NOX2 oxidase, NOX4 oxidase and NOX5 oxidase[19,41]; VSMCs express NOX1 oxidase, NOX4 oxidase and NOX5 oxidase[20,42–44]; and adventitial fibroblasts express NOX2 oxidase and NOX4 oxidase[45–47] (FIG. 2). One study has also reported that NOX2 oxidase is expressed in VSMCs in human small gluteal arteries[48]. This pattern of distribution presumably underlies important but still poorly understood physiological signalling and/or protective roles. As vascular NADPH oxidases, especially NOX2 oxidase, are similar in structure to the phagocytic NADPH oxidase (see below and REFS 49,50 for reviews), it is plausible that their localization — particularly in the inner and outer cell layers of the artery wall — normally represents a means for preventing the transfer of pathogens between the bloodstream and surrounding tissues.

Nevertheless, the expression of the vascular NOX2 subunit and/or its regulatory partners (for example, p47phox or p22phox) is commonly increased by pro-inflammatory stimuli or in the presence of cardiovascular risk factors[51]. The endothelial injury and reduced NO

signalling that occurs in the early stages of vascular disease is largely mediated by excessive levels of NOX2 oxidase-derived superoxide[51,52]. As the NOX2 subunit can generate considerably large amounts of ROS, and pharmacological inhibition or genetic deletion of the NOX2 or p47phox subunits reduces vascular oxidative stress in several disease models[50,53–59] (TABLE 1), there is a strong rationale for therapeutically targeting NOX2 oxidase in the arterial wall for the treatment of vascular disease. Interestingly, in rodents, females appear to have lower levels of vascular NADPH oxidase expression and activity than males or ovariectomized females[60], which could contribute to their lower propensity to develop vascular disease before menopause. It is not yet known whether a similar gender difference exists in humans.

The NOX1 subunit is expressed in endothelial cells and in VSMCs, with most information about its regulation and actions obtained from the latter cell type. In VSMCs, the NOX1 subunit is localized to the plasma membrane, caveolae and endosomes, and it forms a complex with the traditional NADPH oxidase subunits (that is, those subunits utilized by the NADPH oxidase complex originally described in professional phagocytes) p22phox, p47phox and RAC1, as well as with the novel activator subunit NOXA1 (REFS 61–63). The expression of the NOX1 subunit is induced in VSMCs by several substances. These include: vasoactive agonists (such as angiotensin II and thrombin); growth factors (such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor); pro-inflammatory cytokines (such as tumour necrosis factor (TNF) and IL-1β); and atherogenic particles (such as oxidized low-density lipoprotein (LDL) and advanced glycation end products) (for a review, see REF. 20). Moreover, studies in animal models of cardiovascular disease — especially of angiotensin II-induced hypertension — implicate this NADPH oxidase isoform as an important contributor to cardiovascular disease pathology.

First, NOX1 oxidase expression in the vascular wall is elevated in several *in vivo* animal models of hypertension[64–68]. Second, whereas angiotensin II-induced hypertension, vascular superoxide production, endothelial dysfunction and aortic hypertrophy were exacerbated in transgenic mice overexpressing the NOX1 subunit in VSMCs[69,70], most of these parameters were blunted in mice that were globally deficient in the NOX1 subunit[71,72]. Interestingly, mice that were genetically deficient in NOX1 also displayed a marked reduction in aortic expression of type 1 angiotensin II receptors[73], which may explain why angiotensin II-dependent but not noradrenaline-dependent hypertension was sensitive to NOX1 oxidase deficiency[74]. NOX1 oxidase is also implicated in ROS production and in neointimal formation after arterial injury in mice[63,75–77], and the demonstration that expression of the NOX1 and/or NOXA1 subunit is elevated in atherosclerotic lesions of apolipoprotein E-deficient (*ApoE*$^{-/-}$) mice and humans implies a role for the enzyme in this disease state[63,78]. In support of this, there is evidence for a greater reduction in the development of atherosclerotic lesions in the aortic arch of *Nox1*$^{-/-}$/*ApoE*$^{-/-}$ double knockout mice compared to *ApoE*$^{-/-}$ single knockout mice[79].

Although there is substantial evidence linking increased levels of NOX1 and NOX2 oxidases to excessive ROS production in the arterial wall, the roles of the other vascular NADPH oxidase isoforms in vascular disease either remain controversial (in the case of NOX4 oxidase) or ill-defined owing to a lack of rigorous interrogation (in the case of NOX5 oxidase). The NOX4 subunit is highly expressed in endothelial cells, VSMCs and adventitial fibroblasts, and owing to its lack of reliance on traditional cytosolic organizer and activator subunits, NOX4 oxidase activity was, until recently[80], thought to be regulated primarily through changes in expression of its catalytic subunit. Hence, observations that levels of the NOX4 subunit in vascular cells are either downregulated[43,81], unchanged[82] or upregulated[83] — depending on the pathological stimulus under investigation — provide no clear indication of a homeostatic versus pathophysiological function of the catalytic subunit.

Consistent with this, several reports suggest a role for NOX4 oxidase in promoting endothelial cell survival, proliferation and migration[84–87], whereas other studies indicate that NOX4 oxidase mediates effects such as endoplasmic reticulum stress, oxidative DNA damage and apoptotic and necrotic cell death of endothelial cells[83,88–90]. Likewise, in VSMCs, NOX4 oxidase was initially described as a key regulator of cellular differentiation and quiescence[77,91] — which is suggestive of a homeostatic function — but was subsequently shown to contribute to VSMC proliferation, migration and hypertrophy under certain conditions[92,93], all of which are important in arterial remodelling and atherogenesis. Finally, even the fact that NOX4 oxidase generates hydrogen peroxide — which is capable of activating pro-inflammatory signalling cascades[7] — rather than superoxide[37,38,94] raises questions as to whether NOX4 oxidase is likely to have a protective or detrimental role in vascular disease.

Although one study suggested that hydrogen peroxide may impair endothelial NO production via phosphorylation of a crucial tyrosine residue (Tyr657) on eNOS[95], other studies have shown that hydrogen peroxide increases eNOS expression and activity[96,97] and may itself act as a vasodilator in certain vascular beds[98–100]. Indeed, this latter action could explain why mice with targeted endothelial overexpression of the NOX4 subunit displayed lower basal blood pressures and improved endothelium-dependent vasodilator function[74].

Although the NOX5 subunit was first shown to be expressed in a human endothelial cell line in 2007, its apparent absence from the rodent genome[101] has contributed to a relative paucity of additional information since this time about its role in vascular physiology and pathophysiology. Unlike other NADPH oxidases, NOX5 oxidase does not appear to interact with any of the known NADPH oxidase regulatory subunits and is likely to function as a stand-alone protein. NOX5 oxidase also has a unique amino terminal Ca^{2+}-binding domain containing four EF hand motifs, which sets it apart from other NADPH oxidases. This domain allows NOX5 oxidase activity to be regulated by increases in cytosolic Ca^{2+} concentration[102], which could be important in chronic vascular diseases in which cytosolic Ca^{2+} concentrations in vascular cells are likely to be elevated. Indeed, in human microvascular endothelial cell lines and primary aortic VSMCs, NOX5 oxidase activity was augmented by relevant pro-atherogenic stimuli, including thrombin, PDGF, angiotensin II and endothelin 1, and in each instance NOX5 oxidase activity was suppressed following depletion of intracellular Ca^{2+} (REFS 41,103). Endothelial cells and coronary arteries from patients with coronary artery disease displayed greater NOX5 subunit expression and Ca^{2+}-dependent ROS production compared to those from healthy subjects[104]. Importantly, Ca^{2+}-dependent but not Ca^{2+}-independent ROS production was reduced by small interfering RNA against the NOX5 subunit, thus indicating that Ca^{2+}-dependent ROS production is due to NOX5 oxidase activity[104]. Although these data suggest a strong association between NOX5 oxidase activity and coronary artery disease in humans, evidence for a cause–effect relationship is not yet proven and additional work is needed to characterize its physiological and pathological roles in the arterial wall.

NADPH oxidases in leukocytes

NOX2 oxidase in phagocytic cells was the first member of the NADPH oxidase family to be identified[49,105]. NOX2 oxidase-expressing leukocytes classically comprise cells of the innate immune system — that is, neutrophils, monocytes and macrophages[106,107]. Macrophages are the predominant inflammatory cell in atherosclerotic lesions, and are thus a major NADPH oxidase-expressing cell in diseased blood vessels of rodents and primates, including humans[108,109] (FIG. 2). Numerous pro-atherogenic stimuli, including *Chlamydia pneumoniae*[110], oxysterols[111], angiotensin II[112] and oxidized LDL[113] activate NOX2 oxidase in macrophages.

In addition, adaptive immune cells (that is, T lymphocytes) express NOX2 oxidase[114]. NOX2 oxidase activity in circulating T lymphocytes is increased in experimental hypertension[115] and stroke[116], and in the former condition this contributes to the hypertension and endothelial dysfunction that occurs in association with the infiltration of T lymphocytes into perivascular fat (FIG. 2). NOX2 oxidase-derived superoxide from macrophages is also necessary for the induction of regulatory T cells (which suppress effector T cells) and thus modulation of T cell-mediated inflammation[117]; however, this occurs through a different and more complex mechanism. Overall, there is strong evidence that excessively high activity of NOX2 oxidase in leukocytes may contribute to vascular diseases through numerous complex mechanisms.

NADPH oxidases in the brain

All of the major cell types that are present in brain tissue (that is, neurons, astrocytes, microglia and vascular endothelial cells) constitutively express NOX1 oxidase, NOX2 oxidase and NOX4 oxidase[49], which presumably reflects physiological roles for NOX oxidase-derived ROS in brain function. Expression of NOX5 oxidase in the brain has not yet been demonstrated. Many inflammatory and neurotoxic stimuli can increase NADPH oxidase activity in microglia and/or cause NADPH oxidase-dependent neuronal death, and these effects also appear to occur predominantly via NOX2 oxidase activity[49,58,118]. Interestingly, oxidative stress in the brain can contribute to systemic hypertension via a mechanism involving activation of T cells[119]. Recent studies highlight that NOX2 oxidase-derived ROS are generated in the neurons of circumventricular organs in response to circulating angiotensin II, leading to increased sympathetic outflow, which causes hypertension, activation of circulating T cells and increased systemic vascular inflammation[118,120].

Cerebral arteries are different from many non-cerebral arteries in that they have higher levels of NADPH oxidase activity[121,122]. Although — as with most systemic vessels — ROS can increase the contractile tone of cerebral arteries, ROS more typically elicit marked vasorelaxant responses in cerebral vessels[52], which tend to increase local blood flow. Interestingly, NOX2 oxidase-derived ROS appear to be important for the normal signalling that occurs in postsynaptic neurons following N-methyl-D-aspartate (NMDA) receptor activation in association with NO-dependent vasodilatation[123]. Furthermore, mice lacking NOX2 or p47phox subunits have mild deficits in learning and memory[124], as has been suggested to occur in patients with CGD[125]. If NADPH oxidase-derived ROS help to maintain cerebral blood flow and neural function, then great caution will be needed in developing future therapeutic strategies involving inhibition of brain NADPH oxidases in cerebrovascular diseases. Important considerations will include identifying which NADPH oxidases are overactive and in which cells.

Nevertheless, studies using transgenic mice have revealed that excessive levels of NOX2 oxidase-derived superoxide do indeed underlie the oxidative stress and cerebrovascular dysfunction that is associated with angiotensin II-dependent hypertension, hypercholesterolaemia and advanced age[118,126,127]. Extensive NMDA receptor activation leads to superoxide-induced neuronal death that is absent in p47phox-deficient neurons[58] and blocked by the NADPH oxidase inhibitor apocynin (see below), which probably implicates NOX2 oxidase as the source of lethal ROS generation in the brain. Moreover, the accumulation of amyloid-β protein in the brain in Alzheimer's disease can activate NOX2 oxidase, and this effect accounts for the cerebral oxidative stress, neurovascular dysfunction and behavioural deficits that have been observed in a mouse model of Alzheimer's disease[59]. Thus, excessive NOX2 oxidase-derived superoxide levels may represent a link between cerebrovascular dysfunction and cognitive impairment associated with several diseases of advanced age, including Alzheimer's disease[118].

There is evidence from studies on global NOX2 oxidase knockout mice that elevated activity of this NADPH oxidase isoform normally contributes to a worsened outcome following stroke, at least in males[53,55,116,128,129], and that the protective effects of apocynin occur via inhibition of NOX2 oxidase[53–55]. The key cell types involved may include resident brain cells (for example, neurons and microglia) and infiltrating leukocytes[116,129]. There is evidence that NOX4 oxidase-derived ROS mediate TNF-induced cerebral endothelial apoptosis[83] and infarct damage after stroke[130]. However, there is divided evidence as to whether NOX1 oxidase activity might contribute to stroke damage[131,132].

Although NOX4 oxidase and NOX5 oxidase could emerge as validated drug targets from future research, we suggest that the evidence discussed in previous sections points to NOX2 oxidase in several cell types, and to a lesser extent NOX1 oxidase in VSMCs, as the NADPH oxidases that currently represent the most promising therapeutic targets for treating vascular diseases.

Established and novel NADPH oxidase inhibitors

The evidence presented above provides a strong rationale for the use of pharmacological inhibitors of NADPH oxidase to combat oxidative stress and its associated vascular pathologies in cardiovascular diseases. Several compounds are commonly cited in the literature as inhibitors of NADPH oxidase (TABLE 2). Some of these have been the focus of recent comprehensive review articles[133–137] and are thus only briefly discussed here. Of these inhibitors, apocynin and diphenyleneiodonium (DPI) are the most widely studied. Although these compounds have proven to be invaluable pharmacological tools in NADPH oxidase research for over two decades, their lack of selectivity for NADPH oxidases over other enzymes and/or their potential to act as pro-oxidants under certain conditions (in the case of apocynin) is likely to limit their clinical utility. The compound 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) is also recognized as an NADPH oxidase inhibitor. However, it has a very low potency (IC_{50} (half-maximal inhibitory concentration) >1 mM) and off-target effects that include irreversible inhibition of serine proteases, and thus AEBSF has little potential for development into a clinically useful drug. The synthetic peptide Nox2ds-tat (also known as Gp91ds-tat) was designed to penetrate cells and prevent the assembly of the NOX2 oxidase complex (see below). This peptide inhibits NOX2 oxidase activity *in vitro* and reduces parameters of cardiovascular disease in a mouse model of hypertension[57]. However, being a peptide it is unlikely to be orally active or display a suitable pharmacokinetic profile to have widespread utility as a clinical drug.

Numerous studies over the past decade have purported to have identified novel NADPH oxidase inhibitors. However, before any compound can be considered to be a selective NADPH oxidase inhibitor, it must be proven to inhibit ROS production in both NADPH oxidase-expressing cells and in cell-free NADPH oxidase preparations without blocking ROS production from other enzymatic and non-enzymatic sources (for a detailed description of these criteria, see REF. 136). For compounds such as plumbagin and fulvene-5, some or most of these criteria have not yet been established, thus it remains to be determined whether they are true NADPH oxidase inhibitors[138,139] as opposed to ROS scavengers or inhibitors of activation mechanisms upstream of NADPH oxidase activity. By contrast, S17834 blocks NADPH oxidase activity in intact endothelial cells and in isolated endothelial cell membranes without inhibiting ROS generated in a cell-free preparation consisting of hypoxanthine and xanthine oxidase[140]. Furthermore, S17834 suppressed TNF-induced leukocyte adhesion, reduced expression of plasminogen activator inhibitor 1, augmented NO production in endothelial monolayers and displayed atheroprotective properties when it was administered chronically to ApoE- or LDL receptor-deficient mice[140–143].

Triazolopyrimidines, including VAS2870 and VAS3947, have also emerged as promising inhibitors of NADPH oxidase activity. These compounds inhibited NADPH oxidase-derived ROS in several cell lines expressing NADPH oxidases and in primary endothelial and VSMC cultures, but had no effect on ROS generated by xanthine oxidase or on eNOS activity[144–146]. VAS2870 inhibited elevated ROS production and restored endothelium-dependent relaxations in aortas from spontaneously hypertensive rats[82] and limited phenylephrine-induced contractions in rat tail arteries [147]. Most recently, intrathecal administration of VAS2870 to mice following experimental stroke was shown to reduce brain infarct volume and improve neurological outcome[130]. In this study, *Nox4^{-/-}* mice were similarly protected against brain infarction and neurological deficits following stroke, and administration of VAS2870 to these animals conferred no further benefits[130].

Pyrazolopyridine derivatives such as GK-136901, which are structurally related to VAS2870 and VAS3947, were recently identified as potent inhibitors of NOX1 oxidase- and NOX4 oxidase-dependent ROS generation from disrupted cell membrane preparations, with K_i values in the mid-nanomolar range[148]. These compounds were approximately tenfold less active against NOX2 oxidase-dependent ROS production ($K_i > 1$ μM), and had no detectable inhibitory effects against ROS generated *in vitro* by xanthine oxidase[148]. GK-136901 was also highly drug-like in terms of its ease of synthesis, high oral bioavailability, lack of off-target effects against a panel of 135 enzymes (including eNOS), and its *in vivo* pharmacokinetic and safety profiles[148].

ML171 (also known as 2-acetylphenothiazine) was recently identified as a specific NOX1 oxidase inhibitor at nanomolar concentrations, with only marginal activity on other cellular ROS-producing sources — including xanthine oxidase and the other NADPH oxidases[149]. ML171 appears to target the NOX1 catalytic subunit but not its cytosolic regulators (the NOXO1, NOXA1 or RAC1 subunits) and it blocks the NOX1 oxidase-dependent ROS-mediated formation of extracellular matrix-degrading invadopodia in colon cancer cells[149]. Finally, a hydrogen peroxide assay using human embryonic kidney cells overexpressing the NOX4 subunit identified several NOX4 oxidase inhibitors from compounds that contained one of five core structures (oxalyl hydrazides, flavonoids, oxindoles, benzoquinolines or benzothiophenes) [150]. From these data the authors constructed a pharmacophore model of a NOX4 oxidase inhibitor that comprised two hydrogen bond donors and two aromatic rings[150]. Further work is needed to determine the isoform selectivity of compounds based on this pharmacophore model as well as their specificity for inhibition of ROS production by NADPH oxidase versus other sources.

All of the emerging NADPH oxidase inhibitor compounds discussed above were either identified directly by high-throughput screening of compound libraries, or they are derivatives of compounds that were initially identified in such screens. Although many of these compounds appear to hold promise as NADPH oxidase inhibitor drugs, it is interesting to note that none of them displays selectivity for NOX2 oxidase over other NADPH oxidases; on the contrary, some compounds were selective for multiple isoforms other than the NOX2 oxidase.

Based on the evidence presented in previous sections of this Review, we conclude that the NOX1 and NOX2 oxidases currently appear to be the major contributors to vascular oxidative stress in cardiovascular disease, and hence are the most logical therapeutic targets. Therefore, given that other NADPH oxidases are known to have important roles in processes such as postural balance (NOX3 oxidase)[151,152], vasodilatation and cell survival (NOX4 oxidase)[99,100,122,153], and thyroid hormone production (DUOX1 and DUOX2 oxidases)[154], one could argue that in cardiovascular disease therapy, inhibitors displaying a dual selectivity for the NOX1 and NOX2 isoforms over other members of the NADPH oxidase

family will probably offer the greatest benefits in terms of therapeutic efficacy versus side-effect profiles.

Nevertheless, the potential implications of inhibiting any important extravascular effects of NOX1 and NOX2 oxidases must be considered. This is of particular relevance for NOX2 oxidase, given its crucial role in the innate immune response to invading pathogens, and the possibility that any attempts to pharmacologically inhibit NOX2 oxidase will severely compromise a patient's immunological function — analogous to the rare, severe genetic condition CGD (BOX 1). It is therefore reassuring to note that a recent study involving patients with CGD demonstrated that severe illness and poor long-term survival were only evident in individuals whose phagocytic ROS production was more than two orders of magnitude lower than healthy controls[155].

Findings from clinical and animal studies show that NADPH oxidase activity in the vascular wall increases by no more than fivefold during cardiovascular disease. Therefore, we suggest that an optimal therapeutic intervention dually targeting vascular NOX1 and NOX2 oxidases would only need to reduce NADPH oxidase activity by less than one order of magnitude. Hence, by titrating the dose of an NADPH oxidase inhibitor, we predict that it will be feasible to partially inhibit the target NADPH oxidase in the vascular wall to alleviate oxidative stress without affecting long-term immune function. In the following section we outline a rational approach that we believe has the potential to identify new target sites for novel, dual inhibitors of vascular NOX1 oxidase and NOX2 oxidase.

A rational drug design approach

Towards the discovery of dual NOX1 and NOX2 oxidase inhibitors

We contend that the goal of selectively targeting the isoforms of NADPH oxidase that are responsible for the pathophysiology of vascular disease could be achieved via a directed drug discovery approach, starting with an extensive knowledge of the subunit–subunit interactions that are common to the NOX1 and NOX2 oxidase complexes, but not used by the other NADPH oxidase family members.

Because protein–protein interactions often involve binding pockets that are too large to be occupied by small-molecule inhibitors, it will be essential to resolve the three-dimensional structures of each of these interacting domains to identify which protein–protein interactions could represent feasible targets for future drugs.

One could postulate that because the catalytic NOX subunit is what primarily distinguishes each NADPH oxidase isoform, drugs targeting the NOX1 and NOX2 subunits should have the highest likelihood of achieving isoform selectivity. However, it must be noted that despite their diverse amino acid sequences, the functional domains on the different NOX catalytic subunits — which are responsible for binding to NADPH, FAD and haem prosthetic groups — are highly conserved[156] and provide little scope for selective pharmacological targeting. A good example to illustrate this is DPI, which forms a covalent adduct with FAD or on a site within the NOX protein that is adjacent to this moiety, thereby preventing electron flow through the catalytic subunit[157,158]. As a result of this mechanism of action, DPI shows no selectivity towards any isoform of NADPH oxidase and also fails to discriminate between NADPH oxidases and other FAD-containing enzymes — including NOS, xanthine oxidase and cytochrome P450 (REF. 50).

This is not to say that other, hitherto undefined sites on NOX subunits do not have the potential to be exploited by selective inhibitors. However, as NOX subunits are integral membrane proteins[50], resolution of their three-dimensional structure to identify druggable

pockets is likely to represent a major technical challenge. Therefore, we suggest that a feasible strategy with a high potential to yield clinically relevant, isoform-selective lead compounds would be to pharmacologically target the cytosolic regulatory subunits that are used exclusively by the vascular NOX1 and NOX2 oxidases.

The subunit composition of each of the seven isoforms of NADPH oxidase is summarized in TABLE 3. Several of the regulatory subunits used by NOX1 and NOX2 oxidases (including p22phox, NOXA1, NOXO1 and RAC1) are also used to varying degrees by other isoforms of NADPH oxidase (including the NOX3 and NOX4 oxidases). Therefore, agents that target these subunits are unlikely to represent specific inhibitors of NOX1 and NOX2 oxidases. By contrast, p47phox is the only subunit that is used specifically by NOX2 oxidase and by the NOX1 oxidase expressed in VSMCs; that is, interactions between p47phox and both of these two isoforms are reasonably selective in the cell types that are involved in the generation of NADPH oxidase-derived ROS in vascular disease. Moreover, p47phox is a soluble protein[50] for which some aspects of its three-dimensional structure have already been resolved by nuclear magnetic resonance (NMR) and X-ray crystallography, and thus further studies to explore its three-dimensional structure for potential druggable sites are highly feasible.

p47phox as a target for current and future NADPH oxidase isoform-selective inhibitors

The p47phox subunit acts as the 'organizer' protein for the NOX2 oxidase complex, as it is the subunit that is responsible for chaperoning the activator subunit, p67phox, to the membrane-bound catalytic domain[159]. p47phox also serves a similar organizer function for the NOX1 oxidase complex that is expressed in VSMCs, by facilitating the interaction of the cytosolic NOXA1 subunit with the catalytic domain of NOX1 oxidase[62,160] (FIG. 1). To achieve these functions, p47phox contains distinct functional domains that allow it to interact simultaneously with the p67phox or NOXA1 subunits, p22phox, and the NOX1 or NOX2 catalytic subunits as well as with phospholipids in the biological membrane to which the whole complex is anchored (FIG. 3). At least one of these domains on p47phox has already been exploited as a target for the novel, rationally-designed NADPH oxidase inhibitor Nox2ds-tat[57]. We predict that further examination of the physicochemical and structural properties of this functional domain and the remaining functional domains on p47phox will yield new opportunities for the development of highly selective dual NOX1 and NOX2 oxidase inhibitors that could be suitable for the treatment of cardiovascular disease. In the following section we look more closely at the interactions of p47phox with other subunits of the NOX1 and NOX2 oxidase complexes and, where possible, discuss how this information might be translated into the design of novel inhibitor drugs.

The p47phox–p67phox interaction

p47phox interacts directly with an Src homology 3 (SH3) domain on p67phox via a PxxP binding motif contained within a proline-rich region (PRR) near its C terminus (amino acids 360–369)[161–165]. Additional contacts with the SH3 domain of p67phox are made by a domain on p47phox that lies distal to the PRR on its C terminus (amino acids 368–390), and these contacts are important for increasing the affinity of the protein–protein interaction (K_A ~20 nM) and for conferring binding partner specificity[166,167].

The interaction between the p47phox PRR and the p67phox SH3 domain is important, both for the constitutive association of p47phox and p67phox in the cytosol of resting cells and for the p47phox-dependent recruitment of p67phox to the membrane[159]. Hence, disruption of this interaction represents a possible strategy for inhibiting NADPH oxidase activation. It is unclear whether any of the current NADPH oxidase inhibitor drugs act by interfering with the interaction between the p47phox PRR and the p67phox SH3 domain. However, a series

of synthetic peptides based on the PRR of p47phox — but not on PRRs of other proteins — were effective at preventing the binding of p47phox to p67phox, thus providing proof of principle that this interaction may represent a future target for selective NOX2 oxidase inhibitors[162].

As mentioned above, the activation of NADPH oxidase in VSMCs relies on the association of the NOXA1 subunit with the membrane-bound NOX1-p22phox flavocytochrome[62]. Nonetheless, p47phox is still likely to act as the organizer subunit for this enzyme complex[62]. The association of p47phox with the NOXA1 subunit is likely to occur via mechanisms that are similar to those involved in the association of p47phox with p67phox. Examination of the C terminus of the NOXA1 subunit reveals it to be highly homologous to that of p67phox, with respect to the presence of the PxxP binding motif-containing SH3-binding domain, and also in the domain further towards the C-terminal end, which is responsible for increasing the binding affinity and specificity of p67phox for p47phox[168,169]. Moreover, substitution of Arg436 for Trp436 within this C-terminal region results in a complete loss of the interaction between the NOXA1 and p47phox subunits[169]. As such, pharmacological strategies that inhibit the interaction of the p47phox PRR and the p67phox SH3 domain would also be expected to block the interaction between the p47phox and NOXA1 subunits, and would thereby inhibit the production of ROS by NOX1 oxidase in VSMCs.

Intramolecular interactions of the p47phox autoinhibitory region with its SH3 and Phox homology domains

In resting cells, p47phox is folded in on itself such that the bis-SH3 and Phox homology (PX) domains of the protein are ensconced[159]. This closed configuration of p47phox is largely due to intramolecular interactions between its polybasic autoinhibitory region (AIR) and bis-SH3 domain[170–172], and also between its bis-SH3 domain and PX domains[173,174]. Activation of p47phox occurs when serine residues within the AIR are phosphorylated by protein kinase C (PKC) or phosphoinositide 3-kinase (PI3K)[175–178]. This destabilizes the interaction of the AIR with the bis-SH3 domain, allowing p47phox to adopt an open conformation whereby its bis-SH3 and PX domains are free to interact with the p22phox PRR and membrane phospholipids, respectively[159].

Based on the above model, one would predict that preventing phosphorylation of the AIR could lock p47phox in its closed state and thereby inhibit its interaction with the membrane components of the NADPH oxidase enzyme. One strategy for achieving this could be the use of compounds that act upstream of p47phox by inhibiting the activity of PKC and other protein kinases that are responsible for phosphorylating the AIR. A more selective approach may involve direct chemical modification of the sulfhydryl side-groups belonging to the crucial serine residues present within the AIR. Indeed, this may be the mechanism by which apocynin achieves its widely reported inhibitory effects on NADPH oxidase[179].

The p47phox–p22phox intermolecular interaction

A key interaction in the assembly of the active NADPH oxidase complex occurs between the p47phox bis-SH3 domain — which is exposed following phosphorylation of the AIR — and a PRR present on the carboxy terminal of p22phox (amino acids 151–160)[180,181]. As is common for many SH3–PRR interactions, the affinity and specificity of the p22phox PRR for the p47phox bis-SH3 domain are enhanced by a series of amino acids that are located just proximal to the PRR of p22phox (amino acids 161–164)[182]. As proof of its importance to NADPH oxidase activation (and thus its potential as a target site for future drugs), a short synthetic peptide spanning amino acids 159–162 of p22phox was shown to bind to the p47phox bis-SH3 site and inhibit its association with p22phox[183]. Moreover, PR39 — an

endogenous antimicrobial peptide produced by neutrophils — was shown to inhibit the translocation of p47phox to the membrane via a direct interaction with the bis-SH3 domain of the protein[184].

NMR solution structures of the p47phox bis-SH3 domain in complex with peptide fragments of either the p22phox PRR[185,172] or the p47phox AIR[170] have revealed that, in its uninhibited state, the p47phox bis-SH3 domain forms a groove along the surface of the protein. Although the lowest energy structure for this bis-SH3 domain is likely to be too shallow to represent a viable docking site for small-molecule ligands, preliminary data of the relative position and orientation of individual amino acids within this three-dimensional space reveal a single tryptophan residue (Trp193) that is exposed to the solvent (N. Hall, A. Christopoulos and P. Sexton, personal communication). This residue is engaged in minimal interactions with surrounding residues and is thus likely to be highly dynamic in solution. Molecular modelling of feasible alternative p47phox conformations reveals that rotation of Trp193 opens up a deeper groove on the surface of the protein, which is within the size range of known druggable binding pockets (FIG. 4). Although it remains to be determined whether virtual ligand screening with this modified p47phox bis-SH3 structure will identify small molecules with *in vitro* and/or *in vivo* NADPH oxidase inhibitory activity, the above discussion nonetheless highlights that potentially specific molecular targets for drugs may arise as more detailed molecular structural information becomes available.

The p47phox–NOX2 subunit intermolecular interaction

Phage display mapping indicates that p47phox directly interacts with the NOX2 subunit[186]. Three putative p47phox-interacting domains have been identified on the NOX2 subunit, including one on the first predicted intracellular loop (amino acids 86–93), and two additional domains on the cytosolic C-terminal tail (amino acids 450–457 and 554–564)[186]. In the absence of detailed three-dimensional structural information for the NOX2 subunit, it is not possible to determine whether these multiple sites lie adjacent to each other in the folded protein to form a single interaction domain or whether they contact p47phox at separate sites. Interestingly, studies looking for regions on p47phox that interact with the NOX2 subunit have so far identified only one site situated within the AIR (amino acids 323–342)[187,188], which may indicate a single point of contact for the NOX2 subunit on p47phox.

Short synthetic peptides spanning any one of the three p47phox-interacting domains on the NOX2 subunit acted as competitive inhibitors of NADPH oxidase activation in both cell-free systems and in electropermeabilized neutrophils[186,189–191]. Elegant follow-up to this work showed that the addition of a human immunodeficiency virus transactivator of transcription (tat) peptide sequence to one of these NOX2 subunit-docking sequences (Nox2ds) resulted in a compound that was effective at inhibiting NOX2 oxidase activity in intact RAW264.7 cells (an immortalized mouse macrophage cell line) and mouse aortas, and at preventing oxidative stress and its vascular sequelae in animal models of hypertension[57].

An alternative strategy involving the adenoviral-mediated expression of either Nox2ds or a dominant negative p67phox construct in adventitial fibroblasts of the rat carotid artery *in vivo* reduced angioplasty-induced ROS production and neointimal proliferation[192,193]. However, it is unclear where on p47phox the Nox2ds-tat binds. It is also unclear whether the site occupied by Nox2ds-tat is involved in the association of p47phox with the NOX1 subunit in VSMCs and, consequently, whether Nox2ds-tat will be effective at inhibiting ROS production in this cell type. Identification of this site, as well as resolution of its three-dimensional structure, should provide important insight as to whether it may represent a future target for small-molecule dual NOX1 and NOX2 oxidase inhibitors.

The p47phox PX–membrane phospholipid interaction

The PX domain is a conserved sequence of approximately 120 amino acids that is present in p47phox and at least 100 other proteins, including p40phox and NOXO1 subunits[194–196]. As previously mentioned, the p47phox PX domain contains a PRR that is important for maintaining p47phox in its inactive state in resting cells. Detailed mutagenesis studies and X-ray crystallography of the p47phox PX domain have revealed that it also contains two highly basic pockets that bind to membrane phosphoinositides following unfolding of the protein[197,198]. Importantly, these binding pockets are structurally distinct from the one that is present on the p40phox PX domain, thus imparting selectivity towards different phospholipids[198]. The first pocket is relatively large (and therefore probably not an appropriate target for small-molecule drugs) and selectively binds to phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P$_2$) with a K_d of ~38 nM. The second pocket is much smaller and has strong affinities towards anionic phospholipids such as phosphatidic acid or phosphatidylserine[198]. It will be interesting to see whether virtual ligand screening of this pocket yields novel selective dual NOX1 and NOX2 oxidase inhibitors.

Conclusions and perspectives

ROS are compelling targets for the prevention and treatment of vascular diseases. Compounds that inhibit the production of ROS should offer considerable advantages over conventional antioxidants, for which clinical efficacy is likely to have been limited owing to poor bioavailability at the site of disease and paradoxical pro-oxidant effects. The NADPH oxidase family of enzymes, particularly those that contain NOX1 or NOX2 catalytic subunits, are important sources of ROS production in the arterial wall, leukocytes and the brain during vascular disease states. These isoforms of NADPH oxidase rely on their organizer subunit, p47phox, for full activity, thus highlighting this protein as a promising molecular target for therapy. However, although p47phox is important for optimal NADPH oxidase function, the enzyme can be activated (albeit less effectively) in its absence. Given the importance of retaining at least some NADPH oxidase activity for normal immune cell function, p47phox is therefore likely to represent a clinically safer target than other subunits that are absolutely required for enzyme activity. Although several studies have demonstrated protective effects of reputed p47phox inhibitors — including apocynin and Nox2ds-tat — in animal models of hypertension, atherosclerosis and stroke, the clinical translation of such findings is only likely to be achieved with the development of inhibitors with improved efficacy, specificity and pharmacokinetic profiles. Further insight into the molecular structure of p47phox and its interactions with other NADPH oxidase subunits and membrane phospholipids should facilitate the development of the next generation of NADPH oxidase inhibitors.

Acknowledgments

Glossary

Innate immunity	The first line of defense against invading microorgansims. This is mediated by a combination of anatomical barriers (such as

skin and internal epithelial surfaces), physiological barriers (such as raised body temperature), secretory molecules (such as digestive enzymes, complement and interferons) and certain kinds of leukocytes (such as macrophages, neutrophils, mast cells and so on).

Microbicidal burst	The rapid release of reactive oxygen species such as superoxide and hydrogen peroxide by white blood cells (for example, neutrophils and macrophages) to eliminate invading microorganisms from the body.
Oxidative stress	An imbalance between the production and inactivation of reactive oxygen species within a biological system, which often results in redox-sensitive modification of macromolecules — including proteins, nucleotides and lipids — and leads to cellular toxicity.
Professional phagocytes	Cells that are able to engulf (phagocytose) particles such as invading pathogens, dead host cells and cell debris with high efficiency. Such cells include monocytes, macrophages, neutrophils, dendritic cells and mast cells.
Acute cardiovascular events	Cardiovascular end points — such as stroke and heart attacks — that result from the sudden cessation of blood flow through arteries owing to a stenosis or a thromboembolus.
Arterial remodelling	A term used to describe any persistent change in the cross-sectional area of a blood vessel. Remodelling may be outward, in which the vessel expands to accommodate a stenosis, or inward, as a result of medial hypertrophy in hypertension, intimal thickening owing to atherosclerosis, transplant vasculopathy or restenosis after balloon angioplasty.
Thrombosis	The formation of a blood clot within a blood vessel.
Restenosis	The reoccurrence of a stenosis leading to restricted blood flow, occurring at a site on the artery that received angioplasty treatment to clear an initial stenosis.
Atherosclerotic plaque	An accumulation of inflammatory cells — mainly macrophages — and cell debris containing lipids, cholesterol, calcium and fibrous connective tissue, which leads to the enlargement of the artery wall.
Redox state	The reduction or oxidation state of a molecule, which is governed by the number of electrons gained or transferred in a chemical reaction.
Adhesion molecules	Integral membrane proteins including integrins, selectins and certain immunoglobulins that mediate the binding of one cell to other cells or extracellular matrix proteins.
Metalloproteinases	A family of protease enzymes possessing a metal centre (most often Zn^{2+}) as the key functional group in their catalytic site.
Inflammasomes	Multiprotein signalling platforms that are activated in response to pathogens or host-derived stress signals that trigger the

maturation of pro-inflammatory cytokines such as interleukin-1β and interleukin-18.

Cohort studies	An analytical study that follows the same group of people over a long period of time and is often used to assess risk of disease.
Endothelial dysfunction	A shift in the activation state of the endothelium towards reduced production of vasodilator substances, such as nitric oxide, and increased production of pro-inflammatory and prothrombotic mediators.
EF hand motifs	A protein structural domain that is involved in binding Ca^{2+}, and is characterized by a helix–loop–helix topology that has a conformation similar in shape to the spread thumb and forefinger of the hand.
Binding pockets	Regions on a protein that other smaller molecules, termed ligands, can dock onto in a highly specific fashion through the formation of chemical bonds. Such ligands can include endogenous factors such as hormones or neurotransmitters, or exogenous substances such as drugs.
X-ray crystallography	A method of determining the three-dimensional arrangement of atoms within a crystalline substance based on the diffraction pattern generated following bombardment of the crystal with a beam of X-rays.
Phox homology (PX) domain	A conserved protein structural domain that is involved in the targeting of proteins to cellular membranes by binding to phosphoinositides.
Phage display mapping	A method for the study of protein–protein interactions in which proteins of interest are immobilized and exposed to peptide-presenting bacteriophages. Bacteriophages that bind to the protein are used to infect a bacterial host from which the phagemid is collected and the relevant DNA is sequenced and used to predict the primary sequence of the bound peptide.

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Box1 | Chronic granulomatous disease

- Chronic granulomatous disease (CGD)[1,199–202] is a primary immunodeficiency that affects phagocytes of the innate immune system, and is characterized by a markedly increased susceptibility to severe bacterial and fungal infections.

- CGD is caused by any of the >400 mutations that have been identified so far in one of the four genes that encode the subunits of the phagocytic NOX2 subunit-dependent NADPH oxidase complex.

- The incidence of CGD is approximately 1 in 200,000 live births. Most (95%) of the mutations that cause CGD lead to complete or partial loss of protein expression, whereas approximately 5% of mutations are loss-of-function mutations that result in normal levels of protein expression — although with impaired function.

- Most (more than two-thirds) of CGD cases are X-linked recessive and result from defects in the *CYBB* gene that encodes the NOX2 subunit. The remaining cases of CGD are autosomal recessive and caused by defects in the *CYBA, NCF1* and *NCF2* genes, which encode p22phox, p47phox (also known as neutrophil cytosol factor 1) and p67phox (also known as neutrophil cytosol factor 2), respectively. To date, there are no reports of CGD caused by defects in the gene encoding a fifth NADPH oxidase subunit, p40phox. One patient has been identified with a related immunodeficiency resulting from a defect in the gene that encodes the small GTPase RAC2.

- Because the disease is often X-linked, female carriers of genes with mutations that lead to CGD may have either one or no normal copies of the affected gene, whereas unaffected female subjects usually have two normal copies of the gene. Importantly, this so-called gene-dosing effect has identified that despite a reduced level of NADPH oxidase activity, there is no CGD pathology in female subjects with only one copy of causative genes.

- A recent study in patients with CGD demonstrated that severe illness and poor long-term survival was only evident in individuals whose phagocytic ROS production was more than two orders of magnitude lower than in healthy controls[155].

- The above two points may be regarded as indirect evidence that it is feasible to reduce — rather than abolish — NADPH oxidase activity without compromising the innate immune system in patients with cardiovascular risk factors and vascular oxidative stress.

- However, as most previous studies with NADPH oxidase inhibitors have been performed in experimental animals housed under specific pathogen-free conditions, there is a lack of proof of concept — from long-term experimental studies — of the dose-related effects of chronically administered NADPH oxidase inhibitors (for example, apocynin) on infection rate versus oxidative stress.
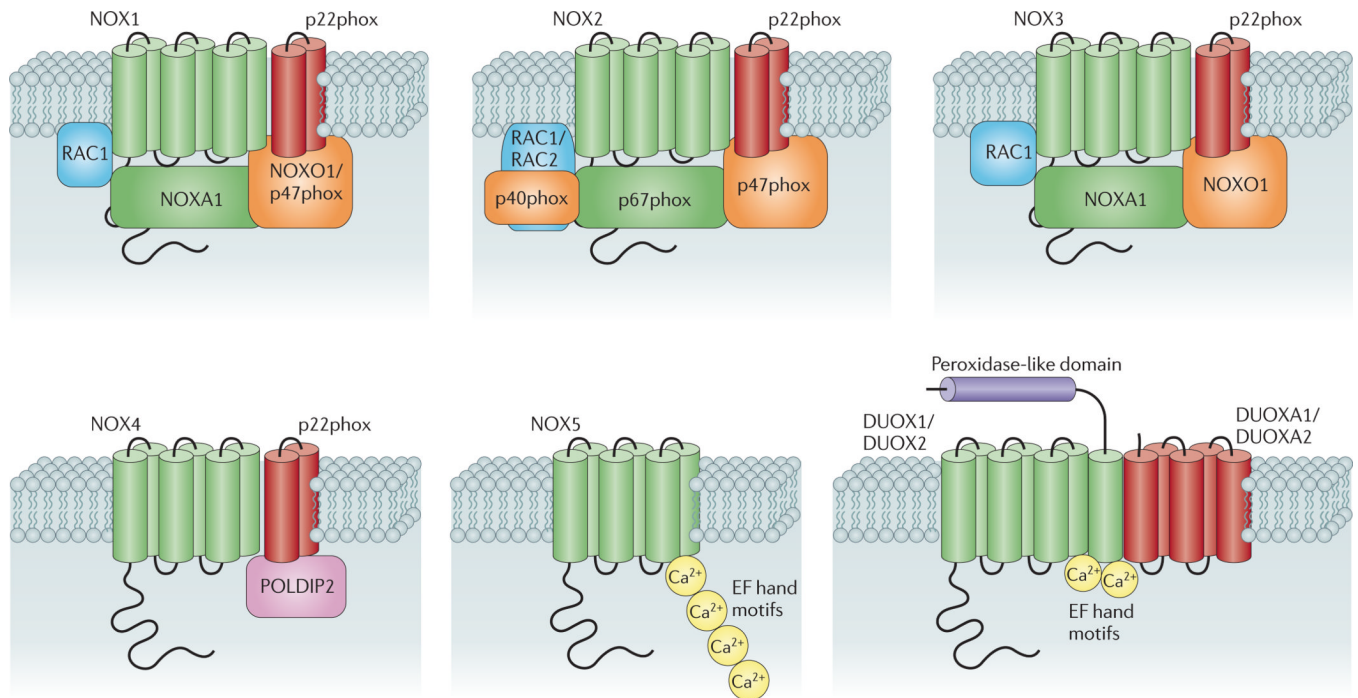
Figure 1. Subunit composition of the seven mammalian NADPH oxidase isoforms
The catalytic core subunits of the enzymes (NADPH oxidase 1 (NOX1)–NOX5, dual oxidase 1 (DUOX1) and DUOX2) are shown in green; NOX and DUOX maturation and stabilization partners (p22phox, DUOX activator 1 (DUOXA1) and DUOXA2) are shown in red; cytosolic organizers (p40phox, NOX organizer 1 (NOXO1) and p47phox) are shown in orange; cytosolic activators (p67phox and NOX activator 1 (NOXA1)) are shown in green; and small GTPases (RAC1 and RAC2) are shown in blue. Also shown (in pink) is polymerase δ-interacting protein 2 (POLDIP2), which is thought to regulate NOX4 activity and link production of reactive oxygen species by this isoform with cytoskeletal organization[80]. EF hand motifs (yellow circles) are also shown, which bind to Ca^{2+} and thereby regulate the activity of NOX5, DUOX1 and DUOX2 oxidases. The figure also illustrates the putative additional amino-terminal transmembrane domain and extracellular peroxidase-like region (shown in purple) on DUOX1 and DUOX2. Although NOX3 oxidase activity is enhanced by the expression of organizer and activator proteins, the enzyme displays constitutive activity in their absence.
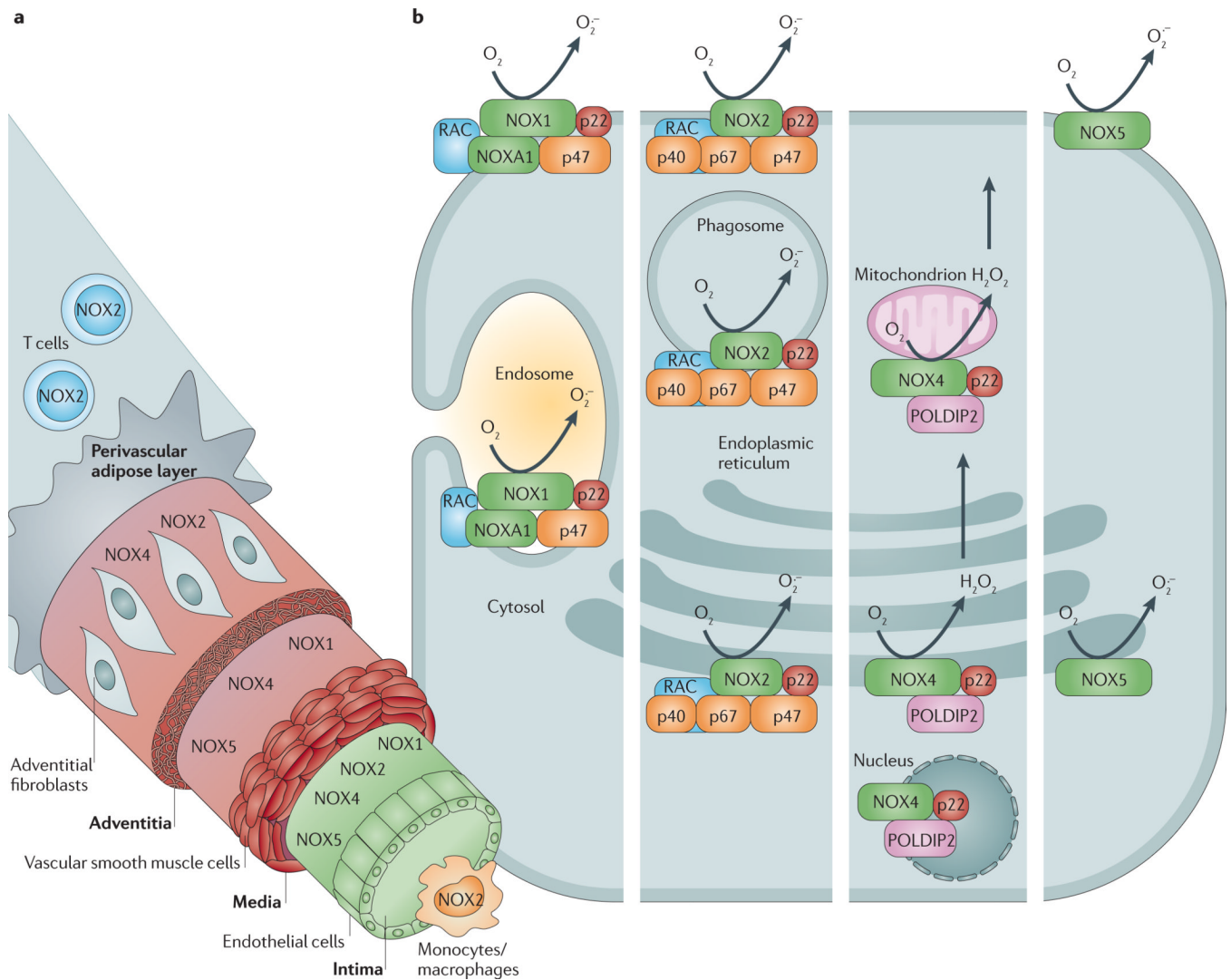
Figure 2. Cellular and subcellular expression of NADPH oxidase isoforms in the blood vessel wall

a | Schematic diagram showing cellular localization (endothelial cells, vascular smooth muscle cells, fibroblasts, macrophages and T cells) of NADPH oxidase isoforms (NOX1 oxidase, NOX2 oxidase, NOX4 oxidase and NOX5 oxidase) through a cross-section of an artery. **b** | Schematic diagram of a hypothetical cell in which all of the vascular NADPH oxidase isoforms (starting with NOX1 oxidase in the left hand column and finishing with NOX5 oxidase in the right hand column) are expressed in each of their possible subcellular locations. H_2O_2, hydrogen peroxide; NOXA1, NADPH oxidase activator 1; $O_2^{\bullet-}$, superoxide; p22, p22phox; p40, p40phox; p47, p47phox; p67, p67phox; POLDIP2, polymerase δ-interacting protein 2.
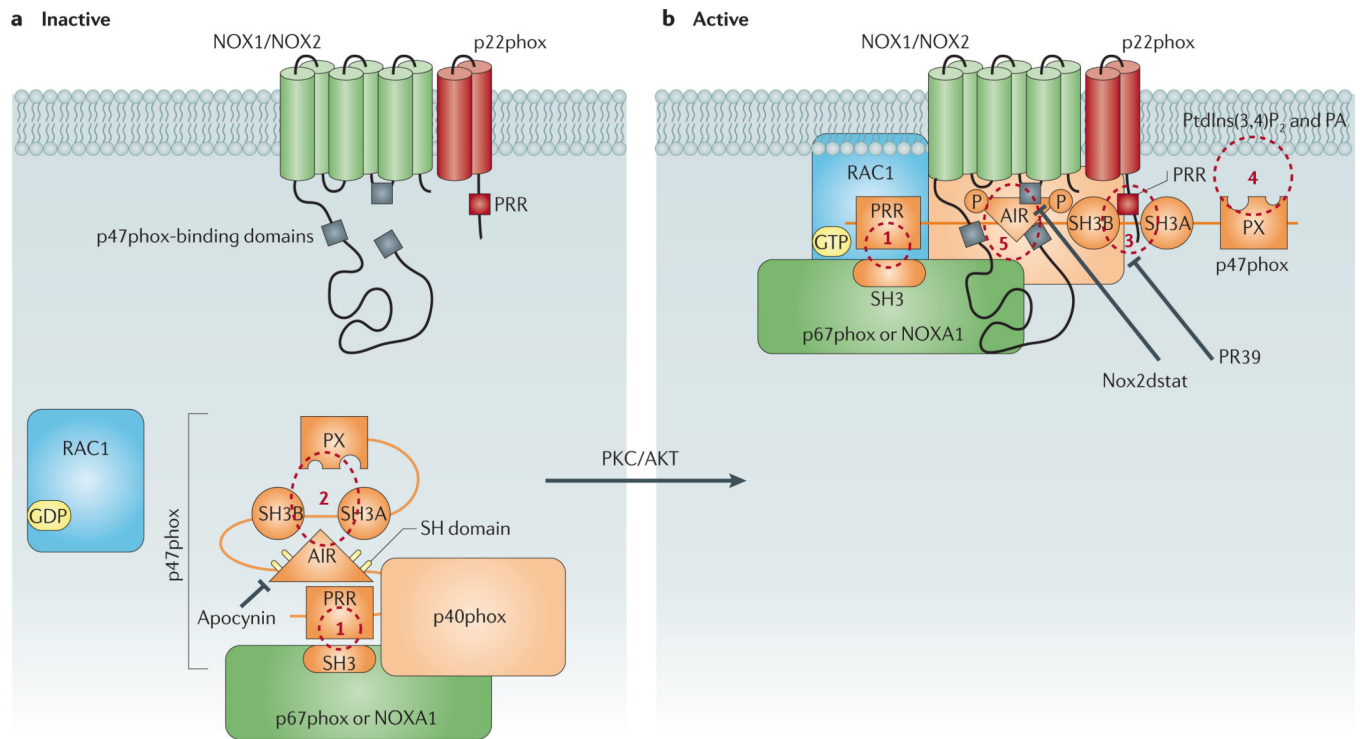
Figure 3. Schematic diagram showing p47phox as the central organizer of the vascular NOX1 and NOX2 oxidases

a | This figure shows the conformation of p47phox in resting cells in which the protein forms a complex with other cytosolic regulatory subunits via an interaction between its carboxy-terminal proline-rich region (PRR) and an Src homology 3 (SH3) domain on one of two potential activator proteins, p67phox or NADPH oxidase activator 1 (NOXA1) subunit (site 1). In the resting state, p47phox adopts a closed conformation in which its tandem-repeat SH3 and phox homology (PX) domains are ensconced and unable to interact with the membrane components. This conformation is largely achieved via intramolecular interactions of both the polybasic autoinhibitory region (AIR) and the PX domain with the tandem-repeat SH3 domain (site 2). **b** | Phosphorylation of sulfhydryl groups (SH) on crucial cysteine residues within the AIR destabilizes these intramolecular interactions and causes p47phox to unfold. This exposes the tandem-repeat SH3 domain of p47phox, thereby allowing it to associate with a PRR on the amino terminus of membrane-bound p22phox (site 3), and the PX domain of p47phox, which interacts with membrane phospholipids including phosphatidylinositol-3,4-biphosphate (PtdIns(3,4)P_2) and phosphatidic acid (PA) (site 4). The association of the cytosolic complex with the membrane subunits is further stabilized by direct protein–protein interactions between p47phox and the NOX2 subunit (site 5). It is unclear whether p47phox undergoes similar protein–protein interactions with the NOX1 subunit. Also shown are likely target sites for conventional NADPH oxidase inhibitors. PKC, protein kinase C.
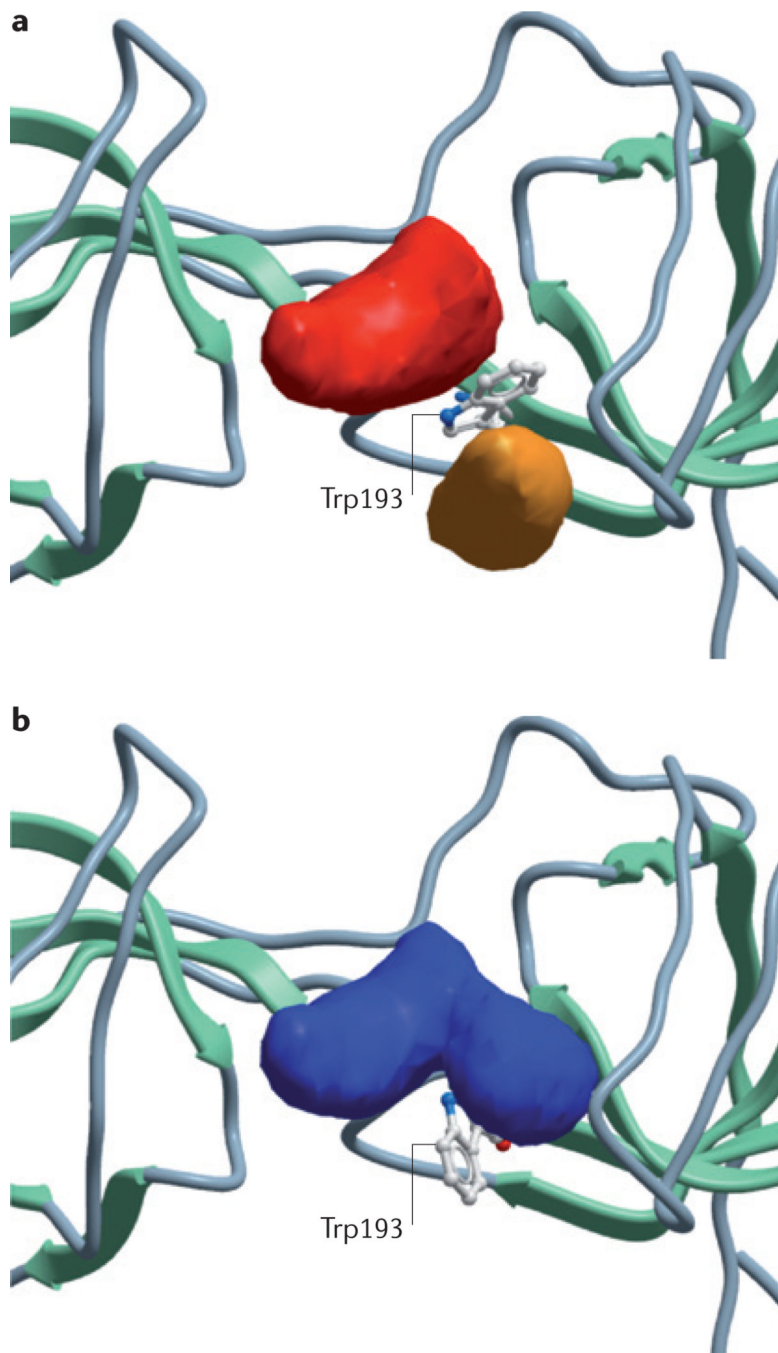
Figure 4. The p47phox tandem-repeat SH3 domain as a potential drug target
Nuclear magnetic resonance solution structure of the p22phox proline-rich region (PRR)-binding domain formed by the tandem repeat Src homology (SH3) domain of p47phox before (**a**) and after (**b**) molecular modelling to rotate a putative dynamic tryptophan residue (Trp193) within its core. Note that rotation of Trp193 results in two smaller pockets (red and orange) combining to form a single large pocket that is potentially druggable.

Table 1

Selected studies of NADPH oxidase in models of vascular disease and stroke
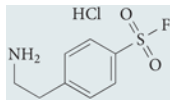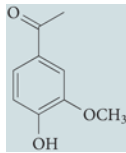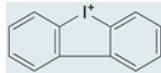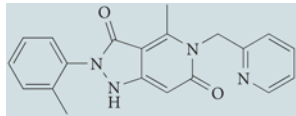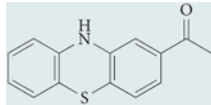
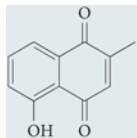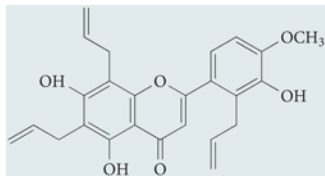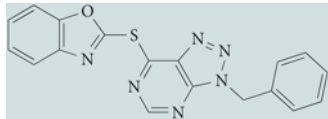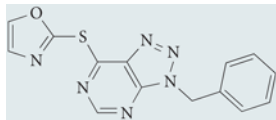| Mouse model	Disease model	Effects	Refs
Hypertension			
Global knockout, *Nox1*$^{-/-}$	Ang II	Decreased BP and $O_2^{•-}$ levels, no change in medial hypertrophy and increased NO bioavailability	72
	Ang II	Decreased BP and $O_2^{•-}$ levels and medial hypertrophy	71
Transgenic, NOX1 overexpression in VSMCs	Ang II	Increased BP and $O_2^{•-}$ levels and medial hypertrophy	69
Double transgenic, *Nox1*$^{-/-}$/TTRhRen	TTRhRen	No change in BP, decreased $O_2^{•-}$ levels and renal fibrosis	203
Global knockout, *Nox2*$^{-/-}$	Ang II	No change in BP, decreased $O_2^{•-}$ levels and 3-nitrotyrosine expression	204
	2K1C	Decreased BP (minimal) and increased NO bioavailability	56
	Ang II	Increased NO bioavailability in afferent renal arterioles, and decreased constrictor responses to Ang II and adenosine	205
Double transgenic, *Nox2*$^{-/-}$/TTRhRen	TTRhRen	No change in BP, decreased $O_2^{•-}$ levels	206
Transgenic, NOX2 overexpression in endothelium	Ang II	Increased BP, increased $O_2^{•-}$ levels in the endothelium only	207
Global knockout, *p47phox*$^{-/-}$	Ang II	Decreased BP and $O_2^{•-}$ levels	208
	DOCA–salt	Decreased BP and $O_2^{•-}$ levels	112
	BMP4	Decreased BP, increased NO bioavailability	209
Adoptive transfer of T lymphocytes from wild-type and *p47phox*$^{-/-}$ mice to *Rag*$^{-/-}$ mice	Ang II	*p47phox*$^{-/-}$ T lymphocytes: decreased BP and $O_2^{•-}$ levels, increased NO bioavailability (versus wild-type T lymphocytes)	115
Atherosclerosis			
Double knockout, *Nox2*$^{-/-}$/*ApoE*$^{-/-}$	*ApoE*$^{-/-}$, HFD	Decreased lesion area in the descending aorta, decreased intimal thickening, decreased $O_2^{•-}$ levels and increased NO bioavailability	81
	ApoE$^{-/-}$, HFD	No change in lesion area in the aortic sinus	210
Double knockout, *p47phox*$^{-/-}$/*ApoE*$^{-/-}$	*ApoE*$^{-/-}$, HFD	No change in lesion area in the aortic sinus	211
	ApoE$^{-/-}$, chow and HFD	No change in lesion area in the aortic sinus, decreased lesion area in the descending aorta	212
	ApoE$^{-/-}$, HFD	Decreased lesion area in the descending aorta (*p47phox*$^{-/-}$ in either vessel-derived or bone marrow-derived cells), decreased oxLDL levels (*p47phox*$^{-/-}$ in bone marrow-derived cells) and decreased adhesion molecule expression (*p47phox*$^{-/-}$ in vessel wall)	213
Restenosis after arterial injury			
Global knockout, *Nox1*$^{-/-}$	Wire-induced injury	Decreased neointimal formation	76
Transgenic, NOX1 overexpression in VSMCs	Wire-induced injury	No effect on neointimal formation	76
Adenoviral-mediated overexpression of NOXA1 in VSMCs	Wire-induced injury	Increased neointimal formation, increased $O_2^{•-}$ levels	63
Global knockout, *Nox2*$^{-/-}$	Wire-induced injury	Decreased neointimal formation, decreased leukocyte accumulation in the neointima	214
Cerebral ischaemia			

Mouse model	Disease model	Effects	Refs
Global knockout, *Nox2*^{-/-}	MCAO	Decreased infarct volume, ICAM1 expression and neutrophil infiltration	54
	MCAO	Decreased infarct volume	55
	MCAO	Decreased infarct volume	128
	MCAO	Decreased infarct volume (*Nox2*^{-/-} mice), no change in infarct volume after bone marrow transplant (wild-type bone marrow to *Nox2*^{-/-} mice, *Nox2*^{-/-} bone marrow to wild-type mice)	129
	MCAO	Decreased infarct volume in males only, decreased $O_2^{\bullet-}$ production by circulating T cells	116
	MCAO	Protective effect of apocynin (decreased infarct volume) absent in *Nox2*^{-/-} mice	53
Global knockout, *Nox1*^{-/-}	MCAO	Increased cortical infarct volume	131
	MCAO	Decreased infarct volume	132
Global knockout, *Nox4*^{-/-}	MCAO	Decreased infarct volume	130

2K1C, two kidneys one-clip; Ang II, angiotensin II; APOE, apolipoprotein E; BMP4, bone morphogenetic protein 4; BP, blood pressure; DOCA, deoxycorticosterone acetate; HFD, high-fat diet; ICAM1, intercellular adhesion molecule 1; MCAO, middle cerebral artery occlusion; NO, nitric oxide; NOX1, NADPH oxidase 1; NOXA1, NOX activator 1; $O_2^{\bullet-}$, superoxide; oxLDL, oxidized low-density lipoprotein; RAG, V(D)J recombination-activating protein; TTRhRen, transgenic mice expressing human renin; VSMC, vascular smooth muscle cell. Table modified from REF. 228.

Table 2

NADPH oxidase inhibitors

Name	Structure	Best characterized mechanism of action relevant to NADPH oxidase	Other pharmacological effects	Refs
AEBSF		Oxidase assembly inhibitor: inhibits association of NOX2 subunit with p47phox. Does not scavenge $O_2^{\bullet-}$ generated in cell-free systems	Nonselective serine protease inhibitor	215
Apocynin		Oxidase assembly inhibitor: inhibits association of p47phox with membrane-bound heterodimer	Scavenger of hydrogen peroxide	216–218
DPI		Flavoprotein inhibitor: abstracts electrons from FAD and prevents electron flow through the flavocytochrome *c*onduit	Inhibitor of NADH-ubiquinone oxidoreductase, NADH dehydrogenase, xanthine oxidase, cytochrome p450 oxidoreductase, NOS and bacterial nicotine oxidase	157,158, 219–223
GK-136901		Purported NOX1 and NOX4 oxidase inhibitor. Mechanism of action not defined, but structural similarity with NADPH suggests that it may act as a competitive substrate inhibitor of this enzyme	None reported	148,224
ML171		A phenothiazine compound with selectivity for NOX1 oxidase (IC_{50} of 0.25 μM) over other NADPH oxidases (IC_{50} >3 μM). Does not scavenge ROS generated by xanthine oxidase activity	None reported	149
Nox2ds-tat	[H]-RKKRRQRRRCSTRIRRQL-NH2	Oxidase assembly inhibitor: inhibits association of NOX2 subunit with p47phox. Does not scavenge $O_2^{\bullet-}$ generated by cell-free systems	None reported	57,188

Name	Structure	Best characterized mechanism of action relevant to NADPH oxidase	Other pharmacological effects	Refs
Plumbagin		Inhibits NAD(P)H-dependent $O_2^{\bullet-}$ production in various cell lines that express NOX4 oxidase; mechanism of action unknown	Napthoquinone structure may impart ROS-scavenging effects	225,226
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP	Binds to SH3 domains of the p47phox subunit and prevents binding to the p22phox subunit	Not selective for NADPH oxidase as it is likely to inhibit other proteins containing SH3 domains	184
S17834		Flavonoid derivative proposed to directly inhibit NADPH oxidase activity, although the mechanism of action is undefined	None reported	140
VAS2870		Undefined mechanism of action; inhibits NADPH oxidase activity in NOX2 oxidase-containing HL-60 cell line and in vascular endothelial cells containing NOX2 and NOX4 oxidases; does not scavenge $O_2^{\bullet-}$	None reported	144,145
VAS3947		A triazolopyrimidine that reduced NADPH oxidase-derived ROS production in several cell lines with low micromolar potency, irrespective of the specific isoforms expressed; showed no inhibitory effects against xanthine oxidase-derived ROS or eNOS activity	None reported	146

eNOS, endothelial nitric oxide synthase; IC$_{50}$, half-maximal inhibitory concentration; NOX, NADPH oxidase; $O_2^{\bullet-}$, superoxide; ROS, reactive oxygen species; SH3, Src homology 3.

Table 3

Comparison of subunit utilization of the seven NADPH oxidases

	NOX1	NOX2	NOX3	NOX4	NOX5	DUOX1	DUOX2
p22phox	+	+	+	+	–	–	–
DUOXA1	–	–	–	–	–	+	+
DUOXA2	–	–	–	–	–	+	+
p67phox	–	+	–	–	–	–	–
NOXA1	+	–	+*	–	–	–	–
p47phox	+‡	+	–	–	–	–	–
NOXO1	+§	–	+*	–	–	–	–
p40phox	–	+	–	–	–	–	–
POLDIP2	ND	ND	ND	+	ND	ND	ND
RAC1	+	+	+	–	–	–	–
RAC2	–	+	–	–	–	–	–

DUOXA1, dual oxidase activator 1; NOXA1, NADPH oxidase activator 1; NOXO1, NOX organizer 1; ND, not determined; POLDIP2, polymerase δ-interacting protein 2.

*
NOX3 is constitutively active when it forms a complex with p22phox; however, its activity is further elevated by NOXA1 and NOXO1 subunits (REF. 227).

‡
Expressed with NOX1 in vascular smooth muscle cells only.

§
Not detectable in vascular smooth muscle cells.