Nox isoforms in vascular pathophysiology: insights from transgenic and knockout mouse models

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Elevated reactive oxygen species (ROS) formation in the vascular wall is a key feature of cardiovascular diseases and a likely contributor to oxidative stress, endothelial dysfunction and vascular inflammation. The NADPH oxidases are a family of ROS generating enzymes, of which four members (Nox1, Nox2, Nox4 and Nox5) are expressed in blood vessels. Numerous studies have demonstrated that expression and activity of at least two isoforms of NADPH oxidase – Nox1 and Nox2 – are up-regulated in animal models of hypertension, diabetes and atherosclerosis. However, these observations are merely suggestive of a role for NADPH oxidases in vessel pathology and by no means establish cause and effect. Furthermore, questions surrounding the specificity of current pharmacological inhibitors of NADPH oxidase mean that findings obtained with these compounds must be viewed with caution. Here, we review the literature on studies utilising genetically-modified mouse strains to investigate the roles of NADPH oxidases in experimental models of vascular disease. While several studies on transgenic over-expressing or knockout mice support roles for Nox1- and/or Nox2-containing oxidases as sources of excessive vascular ROS production and causes of endothelial dysfunction in hypertension, atherosclerosis and diabetes, there are still no published reports on the effects of genetic modification of Nox4 or Nox5 in vascular or indeed any other contexts. Further understanding of the roles of specific isoforms of NADPH oxidase in vascular (patho)physiology should provide direction for future programs aimed at developing selective inhibitors of these enzymes as novel therapeutics in cardiovascular disease.

Keywords: Nox isoforms, vascular pathophysiology, mouse models, ROS

Introduction

It is well established that oxidative stress due to increased production of reactive oxygen species (ROS)

has an important role in the pathogenesis of cardiovascular diseases, including hypertension, atherosclerosis and diabetes. There are several sources of ROS within the vascular wall, including xanthine oxidoreductase, the mitochondrial electron transport chain, uncoupled nitric oxide synthase, cyclooxygenase and cytochrome P450.¹ However, for all of these enzymes, ROS production is a mere by-product of an alternative catalytic reaction that represents their primary function. The only enzymes whose sole

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Received 4 February 2010, manuscript accepted 19 March 2010

identified function is to generate ROS are the nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidases and, over recent years, it has emerged that this family of enzymes are a predominant source of ROS in the vasculature. Observations from *in vitro* and *in vivo* studies have shown that, under pathophysiological conditions, ROS production is elevated and that, importantly, this can be blocked by pharmacological inhibitors of NADPH oxidase, including gp91ds-TAT, apocynin and diphenyleneiodonium (DPI).² However, the lack of selectivity and specificity of these inhibitors means they cannot be used to distinguish the role of a specific Nox isoform. Studies utilising transgenic mice, which either lack or overexpress a particular subunit of the NADPH oxidase complex, have provided direct evidence for a causal role of NADPH oxidases in vascular disease. Here, we will give a brief summary of the structural and functional characteristics of NADPH oxidases expressed in the blood vessel wall and review the literature on studies that have used transgenic and knockout mouse models to investigate the role of NADPH oxidase in vascular disease states including hypertension, atherosclerosis and stroke. Knowledge of the role of specific isoforms of NADPH oxidase in vascular disease is a critical step towards the development of isoform-selective inhibitors which may have potential therapeutic use.

Reactive oxygen species in vascular physiology and pathophysiology

ROS are a family of highly reactive, oxygen-derived molecules that include species such as superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$). All eukaryotic cells generate ROS, partly as a by-product of mitochondrial respiration, but also in a deliberate and tightly regulated fashion for use as signalling molecules. ROS formation begins with the one electron reduction of molecular oxygen (O_2) resulting in the formation of $O_2^{\bullet-}$.^{3,4} $O_2^{\bullet-}$ is then rapidly metabolised to H_2O_2 by a family of enzymes called superoxide dismutases (SOD). H_2O_2 is ultimately metabolised by glutathione peroxidases and catalase into water. The key reactions involved in the formation and metabolism of ROS in cells are summarised in Figure 1.

As mentioned, ROS represent important signalling molecules in eukaryotic cells. H_2O_2 is especially important in this regard through its ability to oxidise, selectively and reversibly, cysteine thiol residues, thereby modifying the activity of various target

Figure 1 Major pathways for ROS formation and breakdown during cell physiology and pathophysiology

proteins such as receptor and non-receptor protein tyrosine kinases, serine threonine kinases, protein tyrosine phosphatases, nuclear transcription factors and ion channels.³ However, in many disease states, including those affecting the vasculature, there is an imbalance between ROS production and their removal by antioxidants. Initially, this imbalance is likely to cause over-activation of redox sensitive signalling pathways leading to cellular 'activation' and a chronic state of inflammation. As the balance shifts even further towards ROS accumulation, however, their higher concentrations in different cellular compartments facilitates their direct chemical reaction with cellular biomolecules. Some of these reactions, such as that between $O_2^{\bullet-}$ and nitric oxide ($NO^{\bullet}$), as well as the Fe^{2+} -catalysed Haber–Weiss reaction between $O_2^{\bullet-}$ and H_2O_2 , lead to the generation of even more powerful oxidising species such as peroxynitrite ($ONOO^-$) and $HO^{\bullet}$, respectively. Myeloperoxidase activity, which is markedly elevated at sites of inflammation, such as at an atherosclerotic plaque, is also an important contributor to the generation of powerful oxidants. This enzyme utilises H_2O_2 as a substrate, along with halides or thiocyanate (SCN^-) to generate hypohalous acids (e.g. $HOCl$) and hypothiocyanate ($HOSCN$), respectively.⁵ These pro-oxidant molecules, along with $ONOO^-$ and $HO^{\bullet}$, further deplete vascular cells of their antioxidant defence mechanisms and may ultimately cause irreversible oxidative damage to cellular proteins, lipids, nucleotides and carbohydrates. This will then result in apoptosis of the affected cells and a local immune response that further exacerbates inflammation and tissue damage. The above processes, characterised by the accumulation of ROS, depletion of antioxidants and oxidative damage to cellular components is often referred to as oxidative stress and is a hallmark of vascular diseases.⁶

The above discussion highlights ROS and/or the enzymes responsible for their excessive generation as

Figure 2 Generalised structures and membrane topologies of the Nox and Duox catalytic subunits of the NADPH oxidase enzymes. All members of the Nox family consist of a cytoplasmic N-terminus, six transmembrane-spanning domains containing four highly conserved histidine residues that form two haem binding sites, and a long C-terminal cytosolic tail containing binding sites for FAD and NADPH. In addition, Nox5 and Duox1–2 have Ca²⁺-binding EF hand motifs while Duox1–2 contain a peroxidase domain. Arrows indicate direction of electron flow through the proteins

potential therapeutic targets to prevent the vascular pathology associated with diseases such as hypertension, diabetes and atherosclerosis. Indeed, several enzyme systems including xanthine oxidoreductase, endothelial nitric oxide synthase (eNOS) and components of the mitochondrial respiratory transport chain have been shown to be important sources of ROS in experimental models and in clinical cases of vascular disease.⁷ Interestingly, for all of these enzymes, ROS production appears to be minimal when the enzyme is functioning normally and only becomes excessive following oxidative depletion of critical co-factors (in the case of eNOS) or oxidative modification of critical protein side chains (in the case of xanthine oxidoreductase and the mitochondrial electron transport chain). This implies that eNOS, xanthine oxidoreductase and mitochondria are not the primary sources of oxidative stress in vascular disease, but that another enzyme(s) is responsible for the initial increase in ROS production that sets in motion the chain of events leading to oxidative stress and tissue damage. Thus, it is now becoming increasingly apparent that NADPH oxidases – a family of enzymes for which the only ascribed

function is the generation of ROS — represent this initial source of ROS.

NADPH oxidases

NADPH oxidase was first identified as the enzyme responsible for the ‘burst’ of ROS generated by professional phagocytes such as neutrophils and macrophages as part of the innate immune response against invading micro-organisms.^{8–10} NADPH oxidases are now known to be a family of multimeric enzyme complexes that generate O₂^{•−} by the transfer of electrons from NADPH to O₂.⁷ The catalytic components of NADPH oxidases reside in their ‘Nox’ or ‘Duox’ subunit. To date, seven members of the NADPH oxidase family have been identified in mammalian tissues and these are defined by the specific Nox (Nox1–5) or Duox (Duox1–2) catalytic subunit they utilise to transfer electrons from NADPH to O₂. Each of the known Nox and Duox proteins are encoded by separate genes and vary considerably in their primary amino acid sequence. Nonetheless, they

Figure 3 Vascular isoforms of NADPH oxidase and their subunit compositions. Nox1, Nox2 and Nox4 require p22phox for activity while Nox1 and Nox2 require additional regulatory subunits. Nox5 does not appear to require other subunits and is activated by Ca²⁺

are all predicted to share fundamentally similar tertiary structures and membrane topologies including a cytoplasmic N-terminus, six transmembrane spanning domains containing four highly conserved histidine residues that form two haem binding sites, and a long C-terminal cytosolic tail containing binding sites for flavin adenine dinucleotide (FAD) and NADPH. The generalised tertiary structures and topologies of the Nox and Duox proteins are depicted in Figure 2.

The Nox5- and Duox1- and Duox2-containing isoforms of NADPH oxidase are thought to be ‘stand-alone’ enzymes in that their activity does not require association with any other proteins. However, electron flow through Nox1, Nox2, Nox3 and Nox4 requires protein–protein interactions with one or more regulatory subunits. All four of these Nox protein isoforms are known to form heterodimers within the plasma and other intracellular membranes with a smaller membrane-bound protein called p22phox. This protein is thought to be required for stabilising the expression of the Nox protein within membranes since down-regulation of p22phox in cells with small interfering RNAs, causes a concomitant decrease of the Nox protein.¹¹ While association with p22phox appears to be all that is required for Nox4 activity, Nox1, Nox2 and Nox3 are further dependent on association with a cytosolic ‘activator’ subunit (p67phox or NoxA1), that interacts with protein

domains on the intracellular loops of the Nox to induce a conformational change that facilitates electron flow; an ‘organiser’ protein (p47phox or NoxO1) that chaperones the activator protein to the membrane; and finally a small GTPase (Rac1 or Rac2) which has been shown to tether p67phox to the membrane and induce a conformational change in p67phox required for Nox activation.^{12,13} The subunit compositions of the different NADPH oxidase isoforms are depicted in Figure 3.

Cellular and subcellular expression of NADPH oxidase isoforms in the blood vessel wall in physiology and pathophysiology

Of the seven mammalian NADPH oxidase isoforms described to date, only Nox1, Nox2, Nox4 and Nox5 appear to be expressed in the constitutive cells of the blood vessel wall (*i.e.* endothelial cells, vascular smooth muscle cells [VSMCs] and adventitial fibroblasts). In addition, immune cells, particularly monocytes, macrophages and T-cells, are known to infiltrate the vessel wall during cardiovascular disease conditions, and these cells express high levels of Nox2. Thus, the remainder of this review will focus only on the four NADPH oxidase isoforms that are likely to be of relevance to vascular physiology and pathophysiology.

Nox2 (initially termed gp91phox)

During physiological conditions, Nox2 expression in the blood vessel wall is largely confined to endothelial cells^{14,15} and adventitial fibroblasts.¹⁶ Although Nox2 may also be expressed in medial VSMCs of some resistance vessels,¹⁷ the medial layer of conduit vessels appears to be devoid of this NADPH oxidase isoform.¹⁸ Studies in cell culture and animal models of cardiovascular disease demonstrate that the expression of Nox2 in endothelial cells and adventitial fibroblasts is up-regulated, along with several of its regulatory subunits, including p47phox and p67phox. In addition, infiltrating monocytes, macrophages and T-cells represent major sources of Nox2 during cardiovascular disease states, as do synthetic VSMCs residing in the neointima of atherosclerotic lesions.¹⁹

Within phagocytic cells, Nox2 is primarily expressed on the plasma membrane, such that it generates $O_2^{\cdot-}$ extracellularly (or within specialised plasma membrane-derived vesicles known as phagosomes). The generation of $O_2^{\cdot-}$ into the extracellular compartment by immune (and endothelial) cells residing in the intima of the blood vessel wall is likely to have major consequences for vascular pathology. Specifically, at this location, $O_2^{\cdot-}$ is ideally placed to react with NO^* as it traverses the interstitial space between the endothelium and underlying vascular smooth muscle. This reaction not

only results in a reduction in the bioavailability and thus the atheroprotective functions of NO^* , but also gives rise to $ONOO^-$, which is likely to exacerbate oxidative stress and further promote vessel disease (Fig. 4).

Unlike phagocytic cells, endothelial cells only express a small proportion of Nox2 on their plasma membrane, possibly in specialised lipid raft domains. Instead, the vast majority of Nox2 in these cells is expressed intracellularly as constitutively assembled functional complexes.¹⁴ More specifically, Nox2 displays a perinuclear distribution in endothelial cells, co-localising with markers of the nuclear and endoplasmic reticulum membranes.^{14,15} It is unclear exactly what the functional role of Nox2 at these sites is during normal physiology, or indeed what consequences augmented-expression of Nox2 on intracellular membranes may have in the context of vascular disease. To our knowledge, no studies have investigated the subcellular localisation of Nox2 within adventitial fibroblasts.

Nox1 (initially termed Mox1 [mitogen oxidase 1])

Nox1 was the first Nox2 homologue to be identified.²⁰ Nox1 is located in the endothelium and medial smooth muscle layer of the blood vessel wall.^{21,22} Structurally, Nox1 is similar to Nox2 in that it is dependent on cytosolic subunits for activation.

Figure 4 Schematic diagram showing the reaction between extracellular Nox2-derived $O_2^{\cdot-}$ and nitric oxide (NO) in the vessel wall during vascular pathophysiology. Under physiological conditions, extracellular $O_2^{\cdot-}$ is likely to be produced in only minor amounts that are rapidly dismutated into H_2O_2 by extracellular superoxide dismutase (ecSOD). However, in pathophysiological conditions the elevated levels of Nox2 in the vessel wall, arising from its up-regulation in endothelial cells and the influx of Nox2-expressing inflammatory cells, leads to markedly higher levels of extracellular $O_2^{\cdot-}$. At this elevated concentration, some $O_2^{\cdot-}$ escapes dismutation and instead reacts with endothelial nitric oxide synthase (eNOS)-derived NO as it traverses the interstitial space between the endothelium and underlying vascular smooth muscle. This leads not only to a reduction in NO bioavailability and thus a loss of its many protective actions, but also to the formation of the powerful oxidant peroxynitrite ($ONOO^-$), which exacerbates oxidative stress and enhances disease progression in the vessel wall

Table 1 Summary of the reviewed studies using knockout and transgenic mouse models to investigate NADPH oxidase in vascular disease

Mouse model	Disease model	Effect	Reference
Hypertension			
Nox1 ^{-/-}	Angiotensin II	↓BP and O ₂ ^{•-} , ↔ medial hypertrophy, ↑NO bioavailability	Matsuno <i>et al.</i> ³⁰
Tg ^{Nox1SMC}	Angiotensin II	↓BP, O ₂ ^{•-} and medial hypertrophy	Gavazzi <i>et al.</i> ⁴³
Nox1 ^{-/-} /TTRhRen	Angiotensin II TTRhRen	↑BP, O ₂ ^{•-} and medial hypertrophy ↔ BP, ↓O ₂ ^{•-} and renal fibrosis	Dikalova <i>et al.</i> ⁴⁴ Yogi <i>et al.</i> ⁴⁵
Nox2 ^{-/-}	Angiotensin II 2K1C Angiotensin II	↔ BP, ↓O ₂ ^{•-} and 3-nitrotyrosine expression ↓BP (minimal), ↑NO bioavailability ↑NO bioavailability in afferent renal arterioles, ↓constrictor responses to angiotensin II and adenosine	Wang <i>et al.</i> ⁴⁶ Jung <i>et al.</i> ⁴⁷ Carlstrom <i>et al.</i> ⁴⁹
Nox2 ^{-/-} /TTRhRen Nox2-Tg (endothelium)	TTRhRen Angiotensin II	↔ BP, ↓O ₂ ^{•-} ↑BP, ↑O ₂ ^{•-} in the endothelium only	Touyz <i>et al.</i> ⁴⁸ Bendall <i>et al.</i> ⁵⁰
p47phox ^{-/-}	Angiotensin II DOCA-salt BMP-4	↓BP and O ₂ ^{•-} ↓BP and O ₂ ^{•-} ↓BP, ↑NO bioavailability	Landmesser <i>et al.</i> ⁵¹ Landmesser <i>et al.</i> ⁵³ Miriya <i>et al.</i> ⁵⁴
Adoptive transfer of T lymphocytes from WT and p47phox ^{-/-} mice to RAG ^{-/-} mice	Angiotensin II	p47phox ^{-/-} T-lymphocytes: ↓BP and O ₂ ^{•-} , ↑NO bioavailability (vs wild-type T-lymphocytes)	Guzik <i>et al.</i> ⁵²
Atherosclerosis			
Nox2 ^{-/-} /ApoE ^{-/-}	ApoE ^{-/-} , HFD	↓Lesion area in the descending aorta, ↓intimal thickening, ↓O ₂ ^{•-} , ↑NO bioavailability	Judkins <i>et al.</i> ⁶⁰
	ApoE ^{-/-} , HFD	↔ Lesion area in the aortic sinus	Kirk <i>et al.</i> ⁶¹
p47phox ^{-/-} /ApoE ^{-/-}	ApoE ^{-/-} , HFD ApoE ^{-/-} , chow and HFD ApoE ^{-/-} , HFD	↔ Lesion area in the aortic sinus Lesion area: ↔ aortic sinus, ↓descending aorta ↓Lesion area descending aorta (p47phox ^{-/-} in either vessel-derived and bone marrow-derived cells), ↓oxLDL (p47phox ^{-/-} in bone marrow - derived cells), ↓adhesion molecule expression (p47phox ^{-/-} vessel wall)	Hsieh <i>et al.</i> ⁵⁸ Barry-Lane <i>et al.</i> ⁵⁷ Vendrov <i>et al.</i> ⁵⁹
Restenosis after arterial injury			
Nox1 ^{-/-} , Tg ^{Nox1SMC}	Wire-induced injury	Neointimal formation: ↓Nox1 ^{-/-} , ↔ Tg ^{Nox1SMC}	Lee <i>et al.</i> ⁶⁵
Adenovirus mediated NoxA1 overexpression	Wire-induced injury	↑Neointimal formation, ↑O ₂ ^{•-}	Niu <i>et al.</i> ⁶⁶
Nox2 ^{-/-}	Wire-induced injury	↓Neointimal formation, ↓leukocyte accumulation into the neointima	Chen <i>et al.</i> ⁶⁷
Stroke			
Nox2 ^{-/-}	MCAO	↓Infarct volume, ICAM-1 expression and neutrophil infiltration	Chen <i>et al.</i> ⁶⁸
	MCAO	↓Infarct volume	Kahles <i>et al.</i> ⁶⁹
	MCAO	↓Infarct volume	Kunz <i>et al.</i> ⁷⁰
	MCAO	↓Infarct volume (Nox2 ^{-/-} mice), ↔ infarct volume after bone marrow transplant (wild-type bone marrow to Nox2 ^{-/-} mice, Nox2 ^{-/-} bone marrow to wild-type mice)	Walder <i>et al.</i> ⁷¹
	MCAO	↓Infarct volume in males only, ↓O ₂ ^{•-} production by circulating T-cells	Brait <i>et al.</i> ⁷³
	MCAO	Protective effect of apocynin (↓infarct volume) absent in Nox2 ^{-/-} mice	Jackman <i>et al.</i> ⁷²
Nox1 ^{-/-}	MCAO	↑Cortical infarct volume	Jackman <i>et al.</i> ⁷⁴

↓ decrease; ↑ increase; ↔ no change.

Homologues of p47phox and p67phox, NoxO1 (Nox organiser 1) and NoxA1 (Nox activator 1), respectively, are known to associate with Nox1 for activity to occur.²³ However, there is also evidence to suggest that Nox1 can interact with p47phox and p67phox to produce ROS.²³ Within VSMCs, Nox1 is reported to be co-localised with p22phox in specialised plasma membrane microdomains called caveolae.^{21,24} While expressed at very low levels under physiological conditions,²⁵ Nox1 mRNA in endothelial and VSMCs is up-regulated by a number of stimuli, including angiotensin II,²⁶ platelet-derived growth factor (PDGF),¹⁸ prostaglandin-F₂-alpha (PGF_{2α}),²⁷ interferon-gamma (IFN-γ)²⁸ and oscillatory shear stress.²⁹ Nox1 expression in the blood vessel wall is also markedly up-regulated *in vivo* in experimental animal models of cardiovascular disease including angiotensin II-induced hypertension in mice,³⁰ streptozotocin-induced diabetes in rats³¹ and hypercholesterolaemia-induced atherosclerosis in mice.⁴

Nox4

Nox4 was first identified in the kidney and, hence, initially termed 'Renox'.³² In the vasculature, Nox4 is expressed in endothelial cells, VSMCs and adventitial fibroblasts.^{18,21,22,25,33} While Nox4 requires p22phox for activity and stabilisation, it differs from Nox2 and Nox1 as it does not require cytosolic subunits for activation.^{34,35} In addition, Nox4 differs from the other Nox homologues in that it preferentially produces H₂O₂ rather than O₂^{•-}.³⁴ The lack of requirement for cytosolic subunits suggests that Nox4 may be constitutively active; indeed, this Nox homologue has been shown to be a major contributor to vascular ROS production under basal conditions.^{25,26} However, Nox4 mRNA can also be up-regulated by various stimuli, including tumour necrosis factor-α (TNF-α),³⁶ IFN-γ²⁸ and lipopolysaccharide.³⁷ Findings from angiotensin II studies appear contradictory as one study reported an increase in Nox4 mRNA,²⁶ while another study found that angiotensin II decreased Nox4 mRNA levels.¹⁸ A number of other factors have also been found to down-regulate Nox4 mRNA such as thrombin, IL-1β and PDGF.^{18,25} Within rat VSMCs, Nox4 has been shown to localise to vinculin within focal adhesions.²¹

Nox5

Nox5 was initially identified in human testis, spleen and kidney³⁸ and is also expressed in vascular endothelial and smooth muscle cells.^{39,40} In addition to the NADPH, FAD and haem binding sites conserved among the Nox proteins, Nox5 has a unique N-terminal calcium binding domain containing four EF hand motifs, allowing its activity to be regulated by

Ca²⁺.³⁸ Nox5 also differs in that p22phox is not essential for activation.^{11,39} Currently, five splice variants of Nox5 have been identified and they are termed Nox5α, Nox5β, Nox5γ, Nox5δ and the short 5s, which lacks the calcium-binding domain.^{38,39} Endothelial cells and VSMCs contain several splice variants and, within endothelial cells, Nox5 is expressed in the perinuclear compartment and is co-localised with markers for the endoplasmic reticulum.³⁹ Nox5 activity is up-regulated in response to thrombin in human microvascular endothelial cells³⁹ and also by PDGF in human aortic VSMCs.⁴⁰ In addition, down-regulation of Nox5 in both these cell types led to decreased cell proliferation in response to thrombin and PDGF, respectively.^{39,40} A recent study by Guzik *et al.*⁴¹ examined Nox5 expression in arteries obtained from coronary artery disease (CAD) patients. Endothelial cells and coronary arteries from CAD patients displayed greater Nox5 expression and Ca²⁺-dependent ROS production compared to healthy subjects. Importantly, Ca²⁺-dependent, but not Ca²⁺-independent, ROS production was reduced by Nox5 small interfering RNA (siRNA), indicating that Ca²⁺-dependent ROS production was due to Nox5 activity.⁴¹ Interestingly, while Nox5 expression was mainly confined to the endothelium in the early stages of atherosclerosis, its expression was significantly increased in smooth muscle underlying complex atherosclerotic lesions.⁴¹ While this study suggests a strong association between Nox5 activity and CAD, evidence for a causal role for Nox5 is still lacking and future experimental studies are hindered by the fact that Nox5 is absent in rodent genomes.

Causal role for Nox isoforms in vascular disease: evidence from transgenic overexpressing and knockout mouse models

The previous section highlights the wealth of literature demonstrating a strong association between several cardiovascular disease states and elevated expression of NADPH oxidases in the vessel wall. This association has been especially well documented for the Nox1- and Nox2-containing isoforms of NADPH oxidase. However, while elevated ROS and oxidative stress are becoming increasingly accepted as major underlying causes of vessel disease, studies showing mere correlations between expression of a given ROS generating enzyme and pathological processes cannot be used as definitive evidence of a cause and effect relationship between the two. For this type of evidence, we must look to interventional

approaches where enzyme activity is modulated either by pharmacological or genetic means. In terms of the former approach, several studies have investigated whether compounds with inhibitory actions against NADPH oxidase afford protection against vascular oxidative stress and markers of vessel pathology in experimental animal models of cardiovascular disease (reviewed by Selemidis *et al.*²). The vast majority of these studies have employed apocynin as the 'gold standard' NADPH oxidase inhibitor and have demonstrated that chronic treatment with this compound can ameliorate several disease parameters including vascular ROS production and oxidative stress, endothelial dysfunction and expression of inflammatory molecules. However, given that the purported molecular target of apocynin is p47phox,⁴² studies with this compound cannot discern between roles for Nox1- and Nox2-containing isoforms of NADPH oxidase. Moreover, apocynin is known to be a powerful scavenger of ROS, including $O_2^{\bullet-}$ and H_2O_2 ; thus, its beneficial effects in cardiovascular disease models could reasonably be attributed to: (i) directly inactivating ROS; (ii) negating the activity of any one of the four NADPH oxidase isoforms expressed in the vessel wall; or (iii) for that matter, any other enzyme system that generates ROS under disease settings.

Therefore, in the case of assigning specific pathophysiological roles for isoforms of NADPH oxidases, we are heavily reliant on studies in which the effects of genetic deletion or transgenic over-expression of different components of the enzyme complex on the progression of vascular disease have been investigated. The following section aims to provide a summary of the information that is available in this area to date (also summarised in Table 1). We will also offer suggestions as to potential future studies on transgenic animals that we perceive to be the most pressing for advancing our understanding of the roles of individual isoforms of NADPH oxidase in cardiovascular disease.

Hypertension

Hypertension is associated with marked elevations in vascular ROS production leading to oxidative stress, endothelial dysfunction and activation of pro-inflammatory pathways within the vessel wall. As such, hypertension is recognised as one of the major risk factors for atherosclerosis and complications thereof, including myocardial infarction and stroke. In mice, hypertension is most commonly induced experimentally by elevating plasma concentrations of angiotensin II, either through subcutaneous infusion of the peptide via an osmotic minipump, transgenic

overexpression of renin, or surgically by renal artery clipping. Indeed, there have been several reports of the effects of genetic manipulation of the NADPH oxidase system in these models.

Two independent studies in mice globally deficient in Nox1 (*i.e.* Nox1^{-/-} mice) showed that both the pressor response and the increase in aortic $O_2^{\bullet-}$ production following chronic angiotensin II infusion were blunted compared to wild-type animals.^{30,43} One of these studies further reported a reduction in the vascular medial hypertrophic response to angiotensin II in Nox1^{-/-} versus wild-type mice,⁴³ although this protective effect was not observed in the second study.³⁰ Furthermore, endothelium-dependent vasorelaxation to acetylcholine in isolated rings of aorta was impaired following angiotensin II infusion in wild-type mice, but was preserved in Nox1^{-/-} mice.³⁰ Consistent with findings in Nox1^{-/-} mice, transgenic overexpression of human Nox1 in VSMCs of mice caused them to display an enhanced blood pressure response to angiotensin II compared to wild-type litter-mate controls.⁴⁴ Overexpression of Nox1 in mice also led to enhanced angiotensin II-induced aortic $O_2^{\bullet-}$ production and medial hypertrophy.⁴⁴ Importantly, the exaggerated blood pressure, vascular ROS and medial hypertrophic responses in transgenic Nox1 over-expressing animals were largely prevented by treatment with the $O_2^{\bullet-}$ scavenging compound, tempol.⁴⁴ A recent study by Yogi *et al.*⁴⁵ on transgenic renin-overexpressing mice demonstrated that, while Nox1 deletion did not prevent the elevation in systolic blood pressure in this disease model, it did ameliorate markers of renal and systemic ROS production, as well as renal fibrosis and activation of pro-inflammatory signalling pathways.

Similar to findings in Nox1-deficient mice, studies in Nox2-deficient mice strongly support a role for this isoform of NADPH oxidase in many of the pathophysiological changes that occur in the vessel wall in the setting of an over-active renin-angiotensin system. For example, Wang *et al.*⁴⁶ demonstrated that, whereas a pressor dose of angiotensin II (3.2 mg/kg/day) caused increases in aortic $O_2^{\bullet-}$ production, 3-nitrotyrosine expression and medial hypertrophy in wild-type mice, it did not do so in Nox2^{-/-} mice. Likewise, renal artery clipping (2K1C) caused marked endothelial dysfunction in aorta of wild-type mice, but had no impact on endothelium-dependent aortic relaxations to acetylcholine in Nox2^{-/-} mice.⁴⁷ However, notwithstanding these profound effects on vascular disease parameters, Nox2 deletion had little or no effect on elevations in systolic blood pressure in either of the above models, or in a further study on transgenic

renin-overexpressing mice.⁴⁸ Thus, there is some evidence that effects of Nox-derived ROS generation in the vessel wall are unrelated to changes in blood pressure during cardiovascular disease.

However, in contrast to findings in the previous model, we and others have evidence that Nox2 may play a role in the blood pressure response to subpressor doses of angiotensin II. Thus, infusion of angiotensin II into mice at a dose of 0.3 mg/kg/day caused a relatively modest increase in systolic blood pressure in wild-type mice (*i.e.* $\Delta 15$ mmHg), as measured by tail cuff plethysmography, but had an even smaller effect in Nox2^{-/-} mice (*i.e.* $\Delta 7$ mmHg).⁴⁹ We have recently confirmed this observation in a preliminary investigation utilising radiotelemetry. Interestingly, the dampened blood pressure response to subpressor doses of angiotensin II in Nox2^{-/-} mice was associated with preserved NO bioavailability in afferent renal arterioles and smaller constrictor responses of the same vessels to angiotensin II and adenosine,⁴⁹ which may suggest that the anti-hypertensive effect of Nox2-deletion in this subpressor angiotensin II model was mediated at the level of the kidney. A recent study in mice that transgenically overexpress Nox2 in the endothelium, further supports a role for this isoform of NADPH oxidase in blood pressure responses to subpressor doses of angiotensin II. Bendall *et al.*⁵⁰ demonstrated that, while 7 days of infusion of angiotensin II at dose of 0.4 mg/kg/day had no effect on systolic blood pressure in wild-type mice, it increased blood pressure by ~ 15 –20 mmHg in the transgenic animals.

Based on the above findings, which indicate that Nox1 and Nox2 act in concert to mediate the pathological effects of angiotensin II in the vessel wall, one might predict that genetic deletion of p47phox (which acts as the organiser subunit for both Nox1 and Nox2 in the vasculature), should have even more profound vasoprotective effects. Indeed, Landmesser *et al.*⁵¹ demonstrated that the hypertensive response to pressor doses of angiotensin II in wild-type mice ($\Delta 45$ mmHg) were markedly blunted in p47phox^{-/-} animals ($\Delta 15$ mmHg), as were the angiotensin II-induced increases in endothelial and VSMC O₂⁻ production in the aorta. An important recent study by Guzik *et al.*⁵² showed that T-cells are likely to be an additional source of p47phox-dependent NADPH oxidase activity in the vessel wall of angiotensin II-treated mice and major contributors to the hypertensive response and vascular pathophysiological actions of this peptide. In that study, RAG1^{-/-} mice, which lack lymphocytes, displayed a reduced pressor response to angiotensin II versus wild-type mice. Interestingly,

while adoptive transfer of T-lymphocytes (but not B-lymphocytes) from wild-type mice restored the angiotensin II-dependent increases in blood pressure, O₂⁻ production, and impairment of endothelium-dependent relaxation to acetylcholine back to the levels seen in wild-type animals, adoptive transfer of T-lymphocytes from p47phox^{-/-} mice did not.⁵²

At least two studies have demonstrated similar protective effects of p47phox deletion in angiotensin II-independent models of hypertension. For example, increases in blood pressure, ROS production and eNOS uncoupling, as well as the impairment in endothelium-dependent vasorelaxation observed in wild-type mice following chronic exposure to deoxycorticosterone acetate (DOCA) salt or bone morphogenic protein-4 (BMP-4), were markedly diminished in p47phox^{-/-} mice.^{53,54}

Overall, the above studies provide convincing evidence for a role for both Nox1- and Nox2-dependent NADPH oxidase activity in vascular ROS production, oxidative stress and endothelial dysfunction irrespective of the magnitude or underlying cause of hypertension. However, the role of these and indeed other isoforms of NADPH oxidase in the actual haemodynamic changes observed in hypertension remain ill-defined.

Atherosclerosis

A major limitation with current hypertension models in mice is that, while they are associated with endothelial dysfunction, medial vascular hypertrophy and activation of pro-inflammatory signalling pathways, they do not lead to the development of true atherosclerotic plaques. Thus, in all of the above studies, it remains unclear whether the reductions in vascular ROS levels and improvements in endothelial function arising from NADPH oxidase deletion would have actually translated to reduced atherogenesis. Thus, we and others have focused our attention on apolipoprotein E-deficient (ApoE^{-/-}) mice. Apolipoprotein E is a glycoprotein that is mainly synthesised in the liver and acts as a ligand for receptors that clear chylomicrons and very low density proteins (VLDLs) from the blood.^{55,56} Thus, ApoE^{-/-} mice have plasma cholesterol levels that are five times higher than wild-type mice and they spontaneously develop fatty streaks and fibrofatty lesions along the aorta and major arterial branches thereof.⁵⁵

Evidence of a role for NADPH oxidase in atherosclerosis was initially obtained in studies using p47phox^{-/-}/ApoE^{-/-} double knockout mice.^{57,58} While two independent studies reported no difference in lesion development between ApoE^{-/-} and p47phox^{-/-}/ApoE^{-/-}

mice at the level of the aortic sinus, it was shown that the absence of p47phox had a profound atheroprotective effect along the length of the descending aorta.^{57,58} Thus, p47phox^{-/-}/ApoE^{-/-} double knockout mice displayed a ~75% reduction in *en face* atherosclerotic lesion area along the descending aorta.⁵⁷ More recently, Vendrov *et al.*⁵⁹ performed allogenic, bone marrow transplantation between ApoE^{-/-} and p47phox^{-/-}/ApoE^{-/-} mice to determine which cells (*i.e.* constitutive cells of the vessel wall versus bone marrow-derived cells) were the source of this pro-atherogenic p47phox-dependent NADPH oxidase activity. These authors concluded that p47phox activity in both vessel wall cells (*e.g.* endothelial cells, VSMCs and/or adventitial fibroblasts) and bone marrow-derived cells (*e.g.* monocytes/macrophages and/or T-cells) contributes to atherogenesis in this model, with the absence of p47phox in either cell type causing a ~50% reduction in *en face* lesion area along the aorta. Interestingly, chimeric ApoE^{-/-} mice lacking p47phox in their bone marrow-derived cells had lower plasma levels of oxidised low-density lipoprotein (ox LDL) compared to the p47phox-intact ApoE^{-/-} mice, while chimeric mice lacking p47phox in the vessel wall displayed decreased expression of cellular adhesion molecules. Hence, this study highlights that, although NADPH oxidase in both bone marrow-derived cells and the vascular wall is important in the pathogenesis of atherosclerosis, the activity of the enzyme in each cell type contributes to disease development via different processes.

As previously mentioned, p47phox is likely to play a critical organiser function for both Nox1- and Nox2-dependent NADPH oxidase activity in the vessel wall. Thus, while the above studies define a role for NADPH oxidase in atherosclerosis they do not provide insight into which specific Nox isoform(s) may be involved. We recently published findings looking at the effect of Nox2 deletion on atherogenesis in ApoE^{-/-} mice.⁶⁰ Consistent with findings in p47phox^{-/-}/ApoE^{-/-} mice^{57,58} and, as reported in an earlier study,⁶¹ we found that the absence of Nox2 had no effect on intimal thickening in the aortic sinus. However, vascular O₂⁻ production along the descending aorta was profoundly lower in Nox2^{-/-}/ApoE^{-/-} versus ApoE^{-/-} mice and this was associated with a marked improvement in NO bioavailability and a dramatic reduction (by ~50%) in *en face* atherosclerotic lesion area.⁶⁰ Thus, it can be concluded that at least some of the protective effects of p47phox deletion in the ApoE^{-/-} model may be attributed to inhibition of the Nox2 isoform of NADPH oxidase.

As for the role of Nox1 in atherogenesis in ApoE^{-/-} mice, preliminary investigations from the group of

Francis Miller (Iowa) are suggestive of a role for this enzyme in the disease process. In a published abstract, Miller and colleagues reported that aortic segments isolated from Nox1^{-/-}/ApoE^{-/-} double knockout mice generated less O₂⁻ and contained fewer lesions, than vessels from ApoE^{-/-} mice.⁶² Moreover, those authors reported that, while lesion development was reduced along the entire length of the aorta, the most profound impact of Nox1 deletion appeared to be in the aortic arch, where *en face* lesion area was ~50% less in Nox1^{-/-}/ApoE^{-/-} versus ApoE^{-/-} mice. Although these preliminary findings need to be confirmed, an especially important role for Nox1 in disease progression in the aortic arch versus the remainder of the aorta is plausible when one considers that this region of the vascular tree is constantly exposed to turbulent blood flow. A previous study demonstrated that oscillatory shear stress over the surface of cultured endothelial cells caused an increase in Nox1 expression, ROS production, ICAM-1 expression and monocyte adhesion.²⁹ Importantly, down-regulation of Nox1 expression with siRNA prevented the oscillatory shear-induced increases in ROS production and monocyte adhesion.²⁹

Restenosis after arterial injury

Restenosis or vessel re-narrowing, is a common complication of percutaneous transluminal coronary angioplasty. Restenosis is associated with the formation of neointimal lesions, which is characterised by extracellular matrix deposition, the migration of VSMCs and myofibroblasts into the intima, and their subsequent proliferation.⁶³ A common mouse model of neointimal formation involves wire insertion into the femoral artery, resulting in mechanical denudation of the endothelium and arterial injury.⁶³

A study by Szocs *et al.*⁶⁴ demonstrated that neointimal formation after rat carotid balloon injury is associated with an increase in O₂⁻ and that Nox1 and Nox4 mRNA expression are up-regulated. Interestingly, while Nox1 mRNA expression was elevated 3–15 days after balloon injury, an increase in Nox4 mRNA expression was not observed until day 15.⁶⁴ This is suggestive of different roles for Nox1 and Nox4 during the response to vascular injury and neointimal formation; Nox1 may contribute to the early phase of lesion development such as cell proliferation, while Nox4 may be involved processes that occur in the later stages, such as cell differentiation.

Lee *et al.*⁶⁵ investigated the role of Nox1 in neointimal lesion formation after wire-induced arterial injury in Nox1^{-/-} mice and mice overexpressing Nox1 in VSMCs (Tg^{Nox1SMC}). Surprisingly, while Nox1

deletion led to a reduction in neointimal lesion formation and cellular proliferation, overexpression of Nox1 in VSMC did not increase neointimal lesions compared to wild-type mice.⁶⁵ In contrast to the latter finding, adenovirus-mediated overexpression of the cytosolic regulator subunit of Nox1, NoxA1, led to a significant increase in neointimal formation, as well as $O_2^{\cdot-}$ in medial VSMCs.⁶⁶ These findings point to a role for VSMC Nox1 in the formation of neointimal lesions, particularly in the early proliferative phase. Importantly, the observation that overexpression of NoxA1 but not of Nox1 enhanced neointimal lesion formation, suggests that NoxA1 has an important role in modulating Nox1 activity, and that expression levels of NoxA1 may be a rate-limiting step in Nox1 activation.

A role for Nox2 in neointimal formation has been demonstrated by Chen *et al.*⁶⁷ Compared to wild-type controls, Nox2^{-/-} mice displayed a 34% reduction in neointimal thickening. Nox2 may also play a role in mediating the vascular inflammatory response associated with neointimal formation, as Nox2 deletion led to a reduction in leukocyte accumulation into the neointima.⁶⁷ As an increase in $O_2^{\cdot-}$ has been observed in the adventitia,⁶⁴ Nox2 expressed in these cells may contribute to neointimal lesion formation.

Stroke

The majority of studies investigating the role of NADPH oxidase in stroke have utilised Nox2 deficient mice. In these studies, ischaemic stroke was induced by middle cerebral artery occlusion (MCAO), followed by 1–3 days of reperfusion. We, along with others, have shown that Nox2^{-/-} mice have a ~40–50% smaller infarct volume compared to wild-type controls,^{68–72} providing strong evidence that Nox2 plays a critical role in ischaemic cerebral damage. In addition, Walder *et al.*⁷¹ used bone marrow transplant experiments between wild-type and Nox2^{-/-} mice to investigate further which source of Nox2 (*i.e.* leukocytes or brain tissue) was responsible for stroke damage. Surprisingly, they found that the absence of Nox2^{-/-} from either the brain or circulating leukocytes did not reduce infarct volume, suggesting that both sources of Nox2 may contribute equally to ischaemic cerebral damage. The study by Chen *et al.*⁶⁸ also found that Nox2 may mediate the post-ischaemic inflammatory process, as Nox2^{-/-} mice that had undergone ischaemia-reperfusion had lower ICAM-1 expression and, consequently, a decrease in neutrophil infiltration. A new paper from our laboratory provides evidence that Nox2-dependent $O_2^{\cdot-}$ production by circulating T-lymphocytes may be augmented after

stroke and contribute to the portion of the brain damage that occurs during the reperfusion phase, but that this may not be significant in females.⁷³ In addition, we have recently shown that, after ischaemic stroke using the MCAO model, Nox1^{-/-} mice had similar total infarct volume and oedema to wild-type mice.⁷⁴ Interestingly, we found that cortical infarct volume was 4-fold greater in Nox1^{-/-} mice compared to wild-type mice, suggesting that perhaps Nox1 may play a role in specifically limiting cortical infarct volume.⁷⁴

Diabetes

To date, no study has used transgenic overexpressing or knockout mouse models to investigate the role of NADPH oxidase in the vascular pathology associated with diabetes. Interestingly though, the widely used diabetic mouse strains C57BL6/J-m db/db and db/+ have a spontaneous mutation in their p47phox gene.⁷⁵ As such, neutrophils isolated from these mice fail to produce $O_2^{\cdot-}$ in response to phorbol-12-myristate-13-acetate despite the presence of other oxidase proteins.⁷⁵ Thus, it would be interesting to determine what effect transgenic expression of a functional p47phox protein would have on the progression of vascular disease in this particular mouse strain. Indeed, the C57BLKS/J db/db mouse strain, which expresses p47phox and has normal oxidase activity, appears to display a more severe diabetic phenotype in comparison to the C57BL6/J-m db/db and db/+ mouse strains.^{75,76}

Human NADPH oxidase knockout: CGD patients

Chronic granulomatous disease (CGD) is a rare congenital immunodeficiency disorder in which professional phagocytes are unable to mount a respiratory burst of $O_2^{\cdot-}$. As a consequence, CGD patients have increased susceptibility to potentially fatal microbial and fungal infections.⁷⁷ The clinical features of CGD are due to genetic mutations in any of four genes encoding for components of NADPH oxidase (Nox2, p47phox, p67phox or p22phox).⁷⁷ Therefore, CGD patients should, in theory, represent ideal subjects to study for a causal role for NADPH oxidase in human vascular disease. In reality, the rarity of CGD in humans (occurrence rate of < 1 in 500,000 individuals in most populations) has severely limited such investigations. However, one recent multicentre study recruited X-linked (X)-CGD patients lacking Nox2 to investigate the role of NADPH oxidase in arterial flow-mediated dilatation (FMD), a marker of NO bioavailability.⁷⁸ Compared to control subjects, X-CGD patients had enhanced

FMD and lower platelet Nox2 expression.⁷⁸ Serum levels of NO metabolites (NOx), measured as an alternative marker of NO bioavailability, were also significantly higher in X-CGD patients compared to healthy subjects.⁷⁸ Importantly, FMD directly correlated with NOx levels and inversely correlated with platelet Nox2 expression, suggesting that the enhanced FMD observed in X-CGD patients was due to an increase in NO bioavailability and that Nox2 may normally limit relaxation of NO-mediated arterial tone by an increase in oxidative stress. Hence, this seminal study provides the first direct evidence that NADPH oxidase activity, even in human subjects with no CAD, may lower NO bioavailability and, thus, predispose blood vessels to the development of vascular disease.

Conclusions and future perspectives

Studies employing transgenic overexpression or knockout approaches to investigate the roles of NADPH oxidase in vascular disease strongly implicate Nox1- and Nox2-containing isoforms of the enzyme as major contributors to the process via their roles as mediators of elevated ROS production in the blood vessel wall. This, in turn, triggers other disease processes including endothelial dysfunction, VSMC proliferation, activation of pro-inflammatory signalling events, as well as oxidative stress and damage to cellular and extracellular macromolecules, including LDL, which together contribute to the development of atherosclerosis and/or vessel stenosis. Nevertheless, it must be stressed that, in virtually all of these studies, mice were deficient or over-endowed with the gene of interest from the time of birth. Thus, it is possible that compensatory changes in expression of other NADPH oxidase subunits and isoforms could have occurred during development that may have dampened the true effects of the genetic modification. Hence, there is an urgent need to investigate vascular disease progression in conditional transgenic models, where the expression of a given Nox subunit(s) can be turned on or off at pre-determined times during disease development and in defined cell types. In addition, there is still no published information on the phenotypic effects of transgenic manipulation of Nox4 or Nox5 in mice and it is thus not possible to draw any firm conclusions about the roles of these isoforms in the pathogenesis of vascular disease. For Nox5, there is the added issue that this subunit of NADPH oxidase is not expressed in rodents, meaning that any further advances in our

understanding of its pathogenic role will be reliant on transgenic over-expression studies in rodents or, potentially, gene knockout studies in non-rodent animal models. Finally, while it is still tempting, despite the previously mentioned gaps in knowledge, to speculate that Nox1 and/or Nox2 are likely to represent future therapeutic targets to combat vascular disease, it must be remembered that there are currently no pharmacological inhibitors with efficacy, specificity or isoform selectivity profiles suitable for clinical use. Furthermore, the clinical manifestations of CGD highlight the fact that the complete absence of Nox2-NADPH oxidase activity in immune cells is far more serious than the consequences of its overactivity in the blood vessel wall. Thus, there is still a long way to go to determine whether the clinical cardiovascular benefits of reducing ROS production in the blood vessel wall through more subtle pharmacological modulation of the activity of Nox2, and indeed other isoforms of NADPH oxidase, will outweigh the potential risks associated with inhibition of the extravascular functions of these enzymes.

Acknowledgements

The authors thank the National Health and Medical Research Council of Australia (NHMRC) for salary support to GRD (465109), CGS (350327) and AKW (566711). JR is supported by a stipend jointly funded by the Monash University Faculty of Medicine, Nursing and Health Sciences and the NHMRC (436825).

References

1. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 2007; **87**: 245–313.
2. Selemidis S, Sobey CG, Winkler K, Schmidt HH, Drummond GR. NADPH oxidases in the vasculature: molecular features, roles in disease and pharmacological inhibition. *Pharmacol Ther* 2008; **120**: 254–291.
3. Thomas SR, Witting PK, Drummond GR. Redox control of endothelial function and dysfunction: molecular mechanisms and therapeutic opportunities. *Antioxid Redox Signal* 2008; **10**: 1713–1765.
4. Bengtsson SH, Gulluyan LM, Dusting GJ, Drummond GR. Novel isoforms of NADPH oxidase in vascular physiology and pathophysiology. *Clin Exp Pharmacol Physiol* 2003; **30**: 849–854.
5. Malle E, Marsche G, Arnhold J, Davies MJ. Modification of low-density lipoprotein by myeloperoxidase-derived oxidants and reagent hypochlorous acid. *Biochim Biophys Acta* 2006; **1761**: 392–415.
6. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 2000; **87**: 840–844.
7. Lassegue B, Clempus RE. Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol* 2003; **285**: R277–R297.

8. Babior BM, Lambeth JD, Nauseef W. The neutrophil NADPH oxidase. *Arch Biochem Biophys* 2002; **397**: 342–344.
9. Babior BM, Kipnes RS, Curnutte JT. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J Clin Invest* 1973; **52**: 741–744.
10. Rossi F, Zatti M. Biochemical aspects of phagocytosis in polymorphonuclear leukocytes. NADH and NADPH oxidation by the granules of resting and phagocytizing cells. *Experientia* 1964; **20**: 21–23.
11. Kawahara T, Ritsick D, Cheng G, Lambeth JD. Point mutations in the proline-rich region of p22phox are dominant inhibitors of Nox1- and Nox2-dependent reactive oxygen generation. *J Biol Chem* 2005; **280**: 31859–31869.
12. Hordijk PL. Regulation of NADPH oxidases: the role of Rac proteins. *Circ Res* 2006; **98**: 453–462.
13. Bokoch GM, Zhao T. Regulation of the phagocyte NADPH oxidase by Rac GTPase. *Antioxid Redox Signal* 2006; **8**: 1533–1548.
14. Van Buul JD, Fernandez-Borja M, Anthony EC, Hordijk PL. Expression and localization of NOX2 and NOX4 in primary human endothelial cells. *Antioxid Redox Signal* 2005; **7**: 308–317.
15. Li JM, Shah AM. Intracellular localization and preassembly of the NADPH oxidase complex in cultured endothelial cells. *J Biol Chem* 2002; **277**: 19952–19960.
16. Chamseddine AH, Miller Jr FJ. Gp91phox contributes to NADPH oxidase activity in aortic fibroblasts but not smooth muscle cells. *Am J Physiol* 2003; **285**: H2284–H2289.
17. Touyz RM, Chen X, Tabet F et al. Expression of a functionally active gp91phox-containing neutrophil-type NAD(P)H oxidase in smooth muscle cells from human resistance arteries: regulation by angiotensin II. *Circ Res* 2002; **90**: 1205–1213.
18. Lassegue B, Sorescu D, Szocs K et al. Novel gp91(phox) homologues in vascular smooth muscle cells: Nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circ Res* 2001; **88**: 888–894.
19. Kalinina N, Agrotis A, Tararak F et al. Cytochrome b558-dependent NAD(P)H oxidase-phox units in smooth muscle and macrophages of atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 2002; **22**: 2037–2043.
20. Suh YA, Arnold RS, Lassegue B et al. Cell transformation by the superoxide-generating oxidase Mox1. *Nature* 1999; **401**: 79–82.
21. Hilenski LL, Clemens RE, Quinn MT, Lambeth JD, Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2004; **24**: 677–683.
22. Ago T, Kitazono T, Kuroda J et al. NAD(P)H oxidases in rat basilar arterial endothelial cells. *Stroke* 2005; **36**: 1040–1046.
23. Banfi B, Clark RA, Steger K, Krause KH. Two novel proteins activate superoxide generation by the NADPH oxidase NOX1. *J Biol Chem* 2003; **278**: 3510–3513.
24. Hanna IR, Hilenski LL, Dikalova A et al. Functional association of Nox1 with p22phox in vascular smooth muscle cells. *Free Radic Biol Med* 2004; **37**: 1542–1549.
25. Ellmark SH, Disting GJ, Fui MN, Guzzo-Pernell N, Drummond GR. The contribution of Nox4 to NADPH oxidase activity in mouse vascular smooth muscle. *Cardiovasc Res* 2005; **65**: 495–504.
26. Winkler K, Wunsch S, Kreutz R, Rothermund L, Paul M, Schmidt HH. Upregulation of the vascular NAD(P)H-oxidase isoforms Nox1 and Nox4 by the renin-angiotensin system *in vitro* and *in vivo*. *Free Radic Biol Med* 2001; **31**: 1456–1464.
27. Katsuyama M, Fan C, Yabe-Nishimura C. NADPH oxidase is involved in prostaglandin F2alpha-induced hypertrophy of vascular smooth muscle cells: induction of NOX1 by PGF2alpha. *J Biol Chem* 2002; **277**: 13438–13442.
28. Manea A, Irina Tanase L, Raicu M, Simionescu M. JAK/STAT signaling pathway regulates Nox1 and Nox4-based NADPH oxidase in human aortic smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2010; **30**: 105–112.
29. Sorescu GP, Song H, Tressel SL et al. Bone morphogenic protein 4 produced in endothelial cells by oscillatory shear stress induces monocyte adhesion by stimulating reactive oxygen species production from a nox1-based NADPH oxidase. *Circ Res* 2004; **95**: 773–779.
30. Matsuno K, Yamada H, Iwata K et al. Nox1 is involved in angiotensin II-mediated hypertension: a study in Nox1-deficient mice. *Circulation* 2005; **112**: 2677–2685.
31. Wendt MC, Daiber A, Kleschyov AL et al. Differential effects of diabetes on the expression of the gp91phox homologues nox1 and nox4. *Free Radic Biol Med* 2005; **39**: 381–391.
32. Geiszt M, Kopp JB, Varnai P, Leto TL. Identification of renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci USA* 2000; **97**: 8010–8014.
33. Ago T, Kitazono T, Ooboshi H et al. Nox4 as the major catalytic component of an endothelial NAD(P)H oxidase. *Circulation* 2004; **109**: 227–233.
34. Martyn KD, Frederick LM, von Loehneysen K, Dinauer MC, Knaus UG. Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases. *Cell Signal* 2006; **18**: 69–82.
35. Ambasta RK, Kumar P, Griendling KK, Schmidt HH, Busse R, Brandes RP. Direct interaction of the novel Nox proteins with p22phox is required for the formation of a functionally active NADPH oxidase. *J Biol Chem* 2004; **279**: 45935–45941.
36. Moe KT, Aulia S, Jiang F et al. Differential upregulation of Nox homologues of NADPH oxidase by tumor necrosis factor- α in human aortic smooth muscle and embryonic kidney cells. *J Cell Mol Med* 2006; **10**: 231–239.
37. Park HS, Chun JN, Jung HY, Choi C, Bae YS. Role of NADPH oxidase 4 in lipopolysaccharide-induced proinflammatory responses by human aortic endothelial cells. *Cardiovasc Res* 2006; **72**: 447–455.
38. Banfi B, Molnar G, Maturana A et al. A Ca(2+)-activated NADPH oxidase in testis, spleen, and lymph nodes. *J Biol Chem* 2001; **276**: 37594–37601.
39. BelAiba RS, Djordjevic T, Petry A et al. NOX5 variants are functionally active in endothelial cells. *Free Radic Biol Med* 2007; **42**: 446–459.
40. Jay DB, Papaharalambus CA, Seidel-Rogol B, Dikalova AE, Lassegue B, Griendling KK. Nox5 mediates PDGF-induced proliferation in human aortic smooth muscle cells. *Free Radic Biol Med* 2008; **45**: 329–335.
41. Guzik TJ, Chen W, Gongora MC et al. Calcium-dependent NOX5 nicotinamide adenine dinucleotide phosphate oxidase contributes to vascular oxidative stress in human coronary artery disease. *J Am Coll Cardiol* 2008; **52**: 1803–1809.
42. Stolk J, Hiltermann TJ, Dijkman JH, Verhoeven AJ. Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am J Respir Cell Mol Biol* 1994; **11**: 95–102.
43. Gavazzi G, Banfi B, Deffert C et al. Decreased blood pressure in NOX1-deficient mice. *FEBS Lett* 2006; **580**: 497–504.
44. Dikalova A, Clemens R, Lassegue B et al. Nox1 overexpression potentiates angiotensin II-induced hypertension and vascular smooth muscle hypertrophy in transgenic mice. *Circulation* 2005; **112**: 2668–2676.
45. Yogi A, Mercure C, Touyz J et al. Renal redox-sensitive signaling, but not blood pressure, is attenuated by Nox1 knockout in angiotensin II-dependent chronic hypertension. *Hypertension* 2008; **51**: 500–506.
46. Wang HD, Xu S, Johns DG et al. Role of NADPH oxidase in the vascular hypertrophic and oxidative stress response to angiotensin II in mice. *Circ Res* 2001; **88**: 947–953.
47. Jung O, Schreiber JG, Geiger H, Pedrazzini T, Busse R, Brandes RP. gp91phox-containing NADPH oxidase mediates endothelial dysfunction in renovascular hypertension. *Circulation* 2004; **109**: 1795–1801.
48. Touyz RM, Mercure C, He Y et al. Angiotensin II-dependent chronic hypertension and cardiac hypertrophy are unaffected by gp91phox-containing NADPH oxidase. *Hypertension* 2005; **45**: 530–537.
49. Carlstrom M, Lai EY, Ma Z, Patzak A, Brown RD, Persson AE. Role of NOX2 in the regulation of afferent arteriole responsiveness. *Am J Physiol* 2009; **296**: R72–R79.
50. Bendall JK, Rinze R, Adlam D et al. Endothelial Nox2 overexpression potentiates vascular oxidative stress and hemodynamic response to angiotensin II: studies in endothelial-targeted Nox2 transgenic mice. *Circ Res* 2007; **100**: 1016–1025.
51. Landmesser U, Cai H, Dikalov S et al. Role of p47(phox) in vascular oxidative stress and hypertension caused by angiotensin II. *Hypertension* 2002; **40**: 511–515.
52. Guzik TJ, Hoch NE, Brown KA et al. Role of the T cell in the genesis of angiotensin II induced hypertension and vascular

- dysfunction. *J Exp Med* 2007; **204**: 2449–2460.
53. Landmesser U, Dikalov S, Price SR *et al.* Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 2003; **111**: 1201–1209.
54. Miriyala S, Gongora Nieto MC, Mingone C *et al.* Bone morphogenic protein-4 induces hypertension in mice: role of noggin, vascular NADPH oxidases, and impaired vasorelaxation. *Circulation* 2006; **113**: 2818–2825.
55. Meir KS, Leitersdorf E. Atherosclerosis in the apolipoprotein-E-deficient mouse: a decade of progress. *Arterioscler Thromb Vasc Biol* 2004; **24**: 1006–1014.
56. Plump AS, Smith JD, Hayek T *et al.* Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 1992; **71**: 343–353.
57. Barry-Lane PA, Patterson C, van der Merwe M *et al.* p47phox is required for atherosclerotic lesion progression in ApoE(-/-) mice. *J Clin Invest* 2001; **108**: 1513–1522.
58. Hsich E, Segal BH, Pagano PJ *et al.* Vascular effects following homozygous disruption of p47(phox) : An essential component of NADPH oxidase. *Circulation* 2000; **101**: 1234–1236.
59. Vendrov AE, Hakim ZS, Madamanchi NR, Rojas M, Madamanchi C, Runge MS. Atherosclerosis is attenuated by limiting superoxide generation in both macrophages and vessel wall cells. *Arterioscler Thromb Vasc Biol* 2007; **27**: 2714–2721.
60. Judkins CP, Diep H, Broughton BR *et al.* Direct evidence of a role for Nox2 in superoxide production, reduced nitric oxide bioavailability, and early atherosclerotic plaque formation in ApoE^{-/-} mice. *Am J Physiol* 2010; **298**: H24–H32.
61. Kirk EA, Dinanier MC, Rosen H, Chait A, Heinecke JW, LeBoeuf RC. Impaired superoxide production due to a deficiency in phagocyte NADPH oxidase fails to inhibit atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* 2000; **20**: 1529–1535.
62. Sheehan AL, Takapoo M, Banfi B, Miller Jr FJ. Role for Nox1 NADPH oxidase in atherosclerosis. *Circulation* 2007; **116**: II_244-c.
63. Hui DY. Intimal hyperplasia in murine models. *Curr Drug Targets* 2008; **9**: 251–260.
64. Szocs K, Lassegue B, Sorescu D *et al.* Upregulation of Nox-based NAD(P)H oxidases in restenosis after carotid injury. *Arterioscler Thromb Vasc Biol* 2002; **22**: 21–27.
65. Lee MY, San Martin A, Mehta PK *et al.* Mechanisms of vascular smooth muscle NADPH oxidase 1 (Nox1) contribution to injury-induced neointimal formation. *Arterioscler Thromb Vasc Biol* 2009; **29**: 480–487.
66. Niu XL, Madamanchi NR, Vendrov AE *et al.* Nox activator 1: a potential target for modulation of vascular reactive oxygen species in atherosclerotic arteries. *Circulation* 2010; **121**: 549–559.
67. Chen Z, Keaney Jr JF, Schulz E *et al.* Decreased neointimal formation in Nox2-deficient mice reveals a direct role for NADPH oxidase in the response to arterial injury. *Proc Natl Acad Sci USA* 2004; **101**: 13014–13019.
68. Chen H, Song YS, Chan PH. Inhibition of NADPH oxidase is neuroprotective after ischemia-reperfusion. *J Cereb Blood Flow Metab* 2009; **29**: 1262–1272.
69. Kahles T, Luedike P, Endres M *et al.* NADPH oxidase plays a central role in blood-brain barrier damage in experimental stroke. *Stroke* 2007; **38**: 3000–3006.
70. Kunz A, Anrather J, Zhou P, Orio M, Iadecola C. Cyclooxygenase-2 does not contribute to postischemic production of reactive oxygen species. *J Cereb Blood Flow Metab* 2007; **27**: 545–551.
71. Walder CE, Green SP, Darbonne WC *et al.* Ischemic stroke injury is reduced in mice lacking a functional NADPH oxidase. *Stroke* 1997; **28**: 2252–2258.
72. Jackman KA, Miller AA, De Silva TM, Crack PJ, Drummond GR, Sobey CG. Reduction of cerebral infarct volume by apocynin requires pretreatment and is absent in Nox2-deficient mice. *Br J Pharmacol* 2009; **156**: 680–688.
73. Brait VH, Jackman KA, Walduck AK *et al.* Mechanisms contributing to cerebral infarct size after stroke: gender, reperfusion, T lymphocytes, and Nox2-derived superoxide. *J Cereb Blood Flow Metab* 2010; Epub ahead of print.
74. Jackman KA, Miller AA, Drummond GR, Sobey CG. Importance of NOX1 for angiotensin II-induced cerebrovascular superoxide production and cortical infarct volume following ischemic stroke. *Brain Res* 2009; **1286**: 215–220.
75. Huang CK, Zhan L, Hannigan MO, Ai Y, Leto TL. P47(phox)-deficient NADPH oxidase defect in neutrophils of diabetic mouse strains, C57BL/6J-m db/db and db/+. *J Leukoc Biol* 2000; **67**: 210–215.
76. Hummel KP, Coleman DL, Lane PW. The influence of genetic background on expression of mutations at the diabetes locus in the mouse. I. C57BL-KsJ and C57BL-6J strains. *Biochem Genet* 1972; **7**: 1–13.
77. Heyworth PG, Cross AR, Curnutte JT. Chronic granulomatous disease. *Curr Opin Immunol* 2003; **15**: 578–584.
78. Violi F, Sanguigni V, Carnevale R *et al.* Hereditary deficiency of gp91(phox) is associated with enhanced arterial dilatation: results of a multicenter study. *Circulation* 2009; **120**: 1616–1622.