NADPH oxidases and oxidase crosstalk in cardiovascular diseases: novel therapeutic targets

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Abstract | Reactive oxygen species (ROS)-dependent production of ROS underlies sustained oxidative stress, which has been implicated in the pathogenesis of cardiovascular diseases such as hypertension, aortic aneurysm, hypercholesterolaemia, atherosclerosis, diabetic vascular complications, cardiac ischaemia–reperfusion injury, myocardial infarction, heart failure and cardiac arrhythmias. Interactions between different oxidases or oxidase systems have been intensively investigated for their roles in inducing sustained oxidative stress. In this Review, we discuss the latest data on the pathobiology of each oxidase component, the complex crosstalk between different oxidase components and the consequences of this crosstalk in mediating cardiovascular disease processes, focusing on the central role of particular NADPH oxidase (NOX) isoforms that are activated in specific cardiovascular diseases. An improved understanding of these mechanisms might facilitate the development of novel therapeutic agents targeting these oxidase systems and their interactions, which could be effective in the prevention and treatment of cardiovascular disorders.

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Accumulating evidence indicates that the major enzymatic sources of reactive oxygen species (ROS) in the cardiovascular system are NADPH oxidase (NOX), uncoupled endothelial nitric oxide synthase (eNOS; also known as NOS3), mitochondria and xanthine oxidase (XO)[1]. NOX is distinct from other enzymatic sources because its primary function is to produce ROS. Low levels of ROS produced by certain NOX isoforms (such as NOX2) have been implicated in physiological processes, including cell proliferation, migration, differentiation and cytoskeletal organization[2]. However, excessive production of ROS from activated NOXs contributes to cardiovascular pathogenesis. Of note, NOX-derived ROS, such as superoxide and hydrogen peroxide (H_2O_2), can trigger ROS production through the activation of other enzymatic systems[3–8]. For example, ROS produced from NOX can induce oxidative inactivation of tetrahydrobiopterin (H_4B), an essential cofactor for eNOS, resulting in eNOS uncoupling and the production of superoxide rather than nitric oxide (NO)[9–37]. In addition, ROS can stimulate the conversion of xanthine dehydrogenase (XDH) to XO by oxidation of the sulfhydryl residue. ROS produced by NOX can also cause mitochondrial DNA damage, oxidation of components of the membrane permeability transition pore and opening of the redox-sensitive mitochondrial ATP-sensitive K^+ channel ($mitoK_{ATP}$), all of which contribute to mitochondrial uncoupling and ROS production[1–7,38–42]. Important mechanistic pathways of ROS amplification or propagation to mediate cardiovascular pathogenesis, particularly those centred on NOX-dependent uncoupling of eNOS and consequent mitochondrial dysfunction, are shown in FIG. 1. Indeed, NOX has emerged as the primary oxidase system underlying oxidative stress in vascular diseases, such as hypertension[43], aortic aneurysms[34,44], hypercholesterolaemia[45], atherosclerosis[46,47] and diabetic vascular complications[46,47], as well as in cardiac diseases, including ischaemia–reperfusion (IR) injury[48], myocardial infarction (MI)[49,50], heart failure[51,52] and cardiac arrhythmias[53]. In this Review, we discuss the crosstalk between NOXs and the other ROS-generating systems in the pathogenesis of cardiovascular diseases (CVDs), the targeting of which could reveal novel therapeutic strategies for the treatment and prevention of CVDs.

Oxidases in CVD pathogenesis

NOX family of enzymes

Accumulating evidence indicates that NOXs are the predominant sources of ROS in CVDs[1,5–8,34,43–55]. Genetic modifications of NOX isoforms have specific effects on cardiovascular phenotypes in animal models[26,56–60], indicating a central role of NOXs in the development of CVDs.

Key points

- Activation of NADPH oxidase (NOX) has a critical role in the pathogenesis of cardiovascular diseases.
- Activation of NOX induces activation of downstream secondary oxidase systems, including uncoupled endothelial nitric oxide synthase, dysfunctional mitochondria and xanthine oxidase.
- Crosstalk between oxidases or oxidase systems sustains oxidative stress to mediate the development of cardiovascular diseases.
- Targeting NOXs as well as interactions between NOXs and secondary oxidase systems might be a novel therapeutic strategy for the prevention and treatment of cardiovascular diseases.

Discovery. The first member of the NOX family of enzymes to be discovered was NOX2 (also known as gp91^{phox} or cytochrome b-245 heavy chain); NOX2 was discovered in phagocytes as the enzyme complex underlying the oxidative burst in response to the invasion of microorganisms[61,62]. In 1978, the protein responsible for ROS production in phagocytes was found to be cytochrome b558 (composed of NOX2 and p22^{phox} (also known as cytochrome b-245 light chain))[63,64]. After the successful cloning of NOX2 in 1986, other subunits and isoforms of NOXs were identified and cloned between 1986 and 2006 (REFS[65–84]). So far, seven isoforms of NOXs (NOX1–NOX5, dual oxidase 1 (DUOX1) and DUOX2) have been identified. The historical discovery and characterization of the NOX family oxidases have been thoroughly reviewed previously[85] and are summarized in BOX 1. The development of pharmaceutical inhibitors of the NOXs is summarized in BOX 2, and the latest agents are discussed below. The genetic modification of NOXs in animal models of CVDs is summarized in BOX 3.

Structure. NOXs are multi-transmembrane proteins (NOX1–NOX5 are six-transmembrane proteins, whereas DUOX1 and DUOX2 are seven-transmembrane proteins), with the C-terminus exposed to the cytosol. NOXs share common structural domains, including six conserved transmembrane domains, four conserved haem-binding histidines, the FAD-binding domain and the NADPH-binding domain[80]. NOXs sequentially transfer electrons from NADPH to FAD, haem groups and then to molecular oxygen, leading to superoxide production[86]. Mutation of one proline residue in the NADPH-binding domain inactivates NOX2 (Pro415 in human NOX2)[87], NOX3 (Pro413 in human NOX3)[88] and NOX4 (Pro437 in human NOX4)[60,89], indicating an important role of the NADPH-binding domain in the activation of NOXs. Of note, both *NOX1* and *NOX2* (also known as *CYBB*) are located on chromosome X, whereas other NOX genes are located on autosomes.

The crystal structures of the NOXs have been reported. In 2009, the crystal structure of the N-terminal regulatory domain of a plant NOX in rice (a homologue of mammalian NOX2) was published[90]. Plant NOX proteins have a cytosolic N-terminal region with two EF hands that bind to Ca^{2+} (REF.[90]). These motifs are absent from the mammalian NOX2, but are present in NOX5, DUOX1 and DUOX2 (REF.[90]). In 2017, the crystal structures of the FAD-binding and NADPH-binding domains (known as the C-terminal cytosolic dehydrogenase (DH) domain when combined) of NOX5 from *Cylindrospermum stagnale* were reported[91]. Of note, this DH domain is common to all seven members of the NOX family[91]. The C-terminus was shown to function as a toggle switch and to regulate access of the NADPH to NOX[91]. The structure of the NADPH-binding domain reported in this NOX5 DH domain is very similar to that of the NADPH-binding domain of human NOX2 (REF.[91]) previously deposited in the RCSB Protein Data Bank (ID: 3A1F).

Activation. Each NOX isoform contains one catalytic subunit and other subunits, except for NOX5, which consists of one catalytic subunit alone. As the only membrane-bound subunit, p22^{phox} is required for the stability and activation of NOX1–NOX4 (REF.[92]). Given that NOX2 was the first NOX isoform to be discovered and has been the subject of more mechanistic studies of activation, we first discuss the activation of this isoform. Under resting conditions, NOX2 and p22^{phox} locate at the membrane as an inactive complex, whereas the p40^{phox} (also known as neutrophil cytosol factor 4), p67^{phox} (also known as neutrophil cytosol factor 2) and p47^{phox} (also known as neutrophil cytosol factor 1) subunits are in the cytosol[56,93]. Activation of NOX2 also requires the small GTPase p21-RAC1 (also known as Ras-related C3 botulinum toxin substrate 1) to assemble with NOX2 on the membrane for full activity. Whereas RAC1 is ubiquitously distributed, RAC2 is reportedly required for the activation of NOX2 in differentiated granulocytes derived from the HL60 cell line and in neutrophils[94–96]. Upon NOX2 activation, RAC1 or RAC2 is recruited to the membrane, followed by recruitment of other cytosolic components. p47^{phox} is then phosphorylated by protein kinase C (PKC)[97–99] and translocated to the membrane, together with p67^{phox} and p40^{phox}. Next, phosphorylation of p47^{phox} leads to a conformational change in its structure and subsequent interaction with p22^{phox}, when the tandem SRC homology 3 (SH3) domain in p47^{phox} can bind to the proline-rich region in the cytosolic C-terminus of p22^{phox} (REF.[100]). These assembly processes result in the activation of NOX2. The initial ROS production (especially of H_2O_2) activates proto-oncogene tyrosine-protein kinase Src, leading to epidermal growth factor receptor (EGFR) transactivation and PI3K-dependent activation of RAC1, which further amplifies NOX2 activation[39,101].

NOX1 activation also requires the assembly of multiple subunits[85]. In the process of NOX1 activation, either NADPH oxidase organizer 1 (NOXO1) or p47^{phox} can be phosphorylated by PKC and translocated to the membrane to bind to p22^{phox} (REFS[26,92]). Another difference in NOX1 activation compared with that of NOX2 is the replacement of p67^{phox} with an alternative subunit, NADPH oxidase activator 1 (NOXA1)[85,92].

Owing to the limited expression of NOX3 (only in fetal tissue and the inner ear), the mechanism of NOX3 activation has been studied only in overexpression systems[43,80,92]. Activation of NOX3 reportedly requires p22^{phox} (REFS[88,92]). In the presence of p22^{phox}, NOX3 is active without cytosolic subunits[88,92]. Interestingly, the activity of NOX3 can be increased by RAC1 and the subunits NOXO1–p47^{phox} and NOXA1–p67^{phox} (REFS[88,92]).

The activation of NOX4 does not require cytosolic regulatory subunits other than the membrane partner p22^{phox}. NOX4 is mainly regulated at the expression level[92,102,103]. Polymerase δ-interacting protein 2 (POLDIP2) has been shown to associate with p22^{phox} and to regulate NOX4 activity in vascular smooth muscle cells[104]. POLDIP2 increases NOX4 enzymatic activity and ROS production, leading to increased focal adhesion turnover and vascular smooth muscle cell migration[104].

NOX5 is unique among NOX isoforms in that it contains an N-terminal calmodulin-like domain with four binding sites for Ca^{2+} (EF hands)[105–107]. The activation of NOX5 is Ca^{2+}-dependent and does not require interaction with known subunits[106,108]. In response to an increase in Ca^{2+} concentration, the N-terminus of NOX5 undergoes conformational changes and exposes its hydrophobic patch[105]. This patch provides an interface for intramolecular interaction between the N-terminus

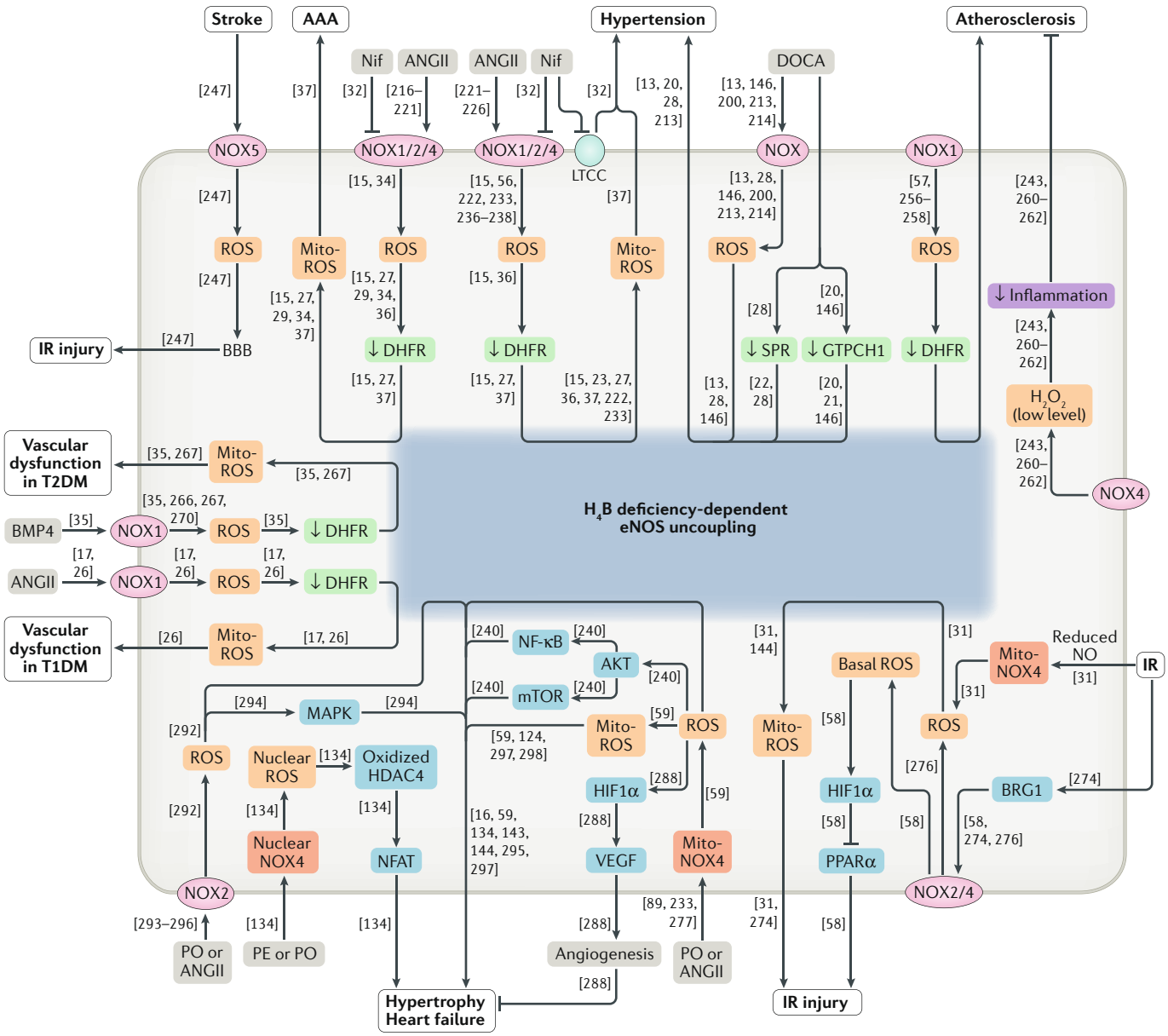

Fig. 1 | **NADPH oxidase-dependent oxidase crosstalk in the pathogenesis of cardiovascular diseases.** NADPH oxidase (NOX)-derived reactive oxygen species (ROS) production induces endothelial nitric oxide synthase (eNOS) uncoupling and mitochondrial dysfunction, resulting in sustained oxidative stress and the development of cardiovascular diseases. Reference numbers are given in square brackets. AAA, abdominal aortic aneurysm; AKT, RACα serine/threonine-protein kinase; ANGII, angiotensin II; BBB, blood–brain barrier; BMP4, bone morphogenetic protein 4; BRG1, transcription activator BRG1; DHFR, dihydrofolate reductase; DOCA, deoxycorticosterone acetate; GTPCH1, GTP cyclohydrolase 1; H_2O_2, hydrogen peroxide; H_4B, tetrahydrobiopterin; HDAC4, histone deacetylase 4; HIF1α, hypoxia-inducible factor 1α; IR, ischaemia–reperfusion; LTCC, L-type calcium channel; MAPK, mitogen-activated protein kinase; Mito, mitochondrial; Mito-ROS, mitochondria-derived reactive oxygen species; mTOR, mechanistic target of rapamycin; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; Nif, nifedipine; NO, nitric oxide; PE, phenylephrine; PO, pressure overload; PPARα, peroxisome proliferator-activated receptor-α; SPR, sepiapterin reductase; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; VEGF, vascular endothelial growth factor.

Box 1 | **Identification of NOXs and subunits**

1960s–1970s

- Presence of oxidase system in phagocytes (1964)[62]
- Cytochrome b558 as a component of the oxidase system (1978)[63,64]

1980s

- Catalytic subunit (now known as NOX2) of NOX in phagocytes (1986)[65]
- $p22^{phox}$ (1987)[66]
- $p47^{phox}$ and $p67^{phox}$ (1988)[67,68]

1990s

- Role of RAC1 and RAC2 (1991)[69,70]
- $p40^{phox}$ (1993)[71]
- NOX in ECs and VSMCs (1994)[72]
- NOX1 (1999–2000)[73,74]

2000s

- NOX3 (2000)[75]
- NOX4 (2000)[76]
- DUOX1 and DUOX2 (2000–2001)[77,78]
- NOX5 (2001)[79,80]
- NOXA1 and NOXO1 (2003)[81–83]
- DUOXA1 and DUOXA2 (2006)[84]

DUOX, dual oxidase; DUOXA, dual oxidase maturation factor; EC, endothelial cell; NOX, NADPH oxidase; NOXA1, NADPH oxidase activator 1; NOXO1, NADPH oxidase organizer 1; VSMC, vascular smooth muscle cell.

and the C-terminus, resulting in the activation of NOX5 (REF.[105]). In the C-terminus, NOX5 has a binding site for the Ca^{2+}-modulated and Ca^{2+}-binding protein calmodulin[109]. Calmodulin is reported to bind to NOX5 in a Ca^{2+}-dependent fashion, resulting in increased Ca^{2+} sensitivity of NOX5 (REFS[109,110]). The activity of NOX5 can also be positively regulated through phosphorylation by PKC (at Ser490, Ser494 and Thr498), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; at Ser475, Thr494, Ser498, Ser502 and Ser659) and mitogen-activated protein kinases (MAPKs; at Ser498)[111–114].

DUOX1 and DUOX2 are composed of the basic NOX5-like structure, but fused with an additional transmembrane domain and an extracellular N-terminus[102,115]. The association of DUOX1 with dual oxidase maturation factor 1 (DUOXA1) and of DUOX2 with DUOXA2 enables the translocation of DUOX1 and DUOX2 from the endoplasmic reticulum to the plasma membrane[102,115]. DUOX1 and DUOX2 are activated by the binding of Ca^{2+} to their intracellular domain[102,115].

The composition of all the NOX isoforms is summarized in FIG. 2. In summary, the activation of NOX1 requires $p22^{phox}$, RAC1, $p47^{phox}$ and/or NOXO1, and NOXA1. The activation of NOX2 requires $p22^{phox}$, RAC1 or RAC2, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$. The activation of NOX4 requires $p22^{phox}$, and the activity of NOX4 can also be regulated by POLDIP2. The activation of NOX5 is primarily dependent on Ca^{2+}. DUOX1 and DUOX2 require DUOXA1 and DUOXA2, respectively, and Ca^{2+} for their activation, and are not expressed in the cardiovascular system.

Subcellular localization. The NOX isoforms each have a specific cellular expression pattern and subcellular localization that determines the types of ROS from each isoform detectable by currently available techniques[116]. NOX1, NOX2, NOX4 and NOX5 are expressed in cardiovascular cells[51,103,117]. Endothelial cells contain NOX1 (REF.[118]), NOX2 (REF.[119]), NOX4 (REF.[118]) and NOX5 (REFS[108,120]). Vascular smooth muscle cells express NOX1 (REF.[73]), NOX4 (REF.[121]) and NOX5 (REFS[79,120]). Cardiomyocytes express NOX1 (REFS[122,123]), NOX2 (REFS[124,125]), NOX4 (REF.[89]) and NOX5 (REF.[126]). The cell-specific expression of NOX isoforms in the cardiovascular system is summarized in FIG. 2.

The subcellular localization of NOX isoforms varies between cell types. NOX2 is localized at the perinuclear cytoskeleton[63] and endoplasmic reticulum[93], whereas NOX4 (REFS[93,127,128]) and NOX5 (REF.[108]) are localized at the endoplasmic reticulum in endothelial cells. NOX1 is localized in the caveolae of vascular smooth muscle cells[129]. Interestingly, NOX4 has been reported to localize in the nucleus, focal adhesions and stress fibres in vascular smooth muscle cells under normal conditions[129–131] and might translocate to the endoplasmic reticulum in hypertension[132]. NOX5 was found in the plasma membrane in vascular smooth muscle cells[47]. In cardiomyocytes, NOX2 is localized in the plasma membrane and the cytosol[125], whereas NOX4 is localized in the mitochondria[133] and nuclei[134].

NOX1, NOX2 and NOX5 produce superoxide directly. Distinct from other NOX isoforms, NOX4 has been shown to produce H_2O_2 through the rapid dismutation of superoxide into H_2O_2 because of a highly conserved histidine residue in NOX4 (REFS[135–137]). Conversely, NOX4 production of H_2O_2 is thought to be the consequence of the localization of NOX4 at the mitochondria in cardiomyocytes and at the endoplasmic reticulum in endothelial cells; superoxide cannot cross the membranes of these subcellular organelles, so only the superoxide dismutated product, H_2O_2, is releasable to the cytoplasm and detectable by currently available methods[138].

Importantly, one of the major consequences of NOX activation is the activation of other oxidase systems to sustain oxidative stress in a process known as ROS-dependent ROS production. These secondary oxidase systems include, but are not limited to, uncoupled eNOS, dysfunctional mitochondria, XO and the endoplasmic reticulum.

Uncoupled eNOS

There are three isoforms of nitric oxide synthase (NOS): eNOS, neuronal NOS (nNOS; also known as NOS1) and inducible NOS (iNOS; also known as NOS2). For the synthesis of NO, L-arginine is required as the substrate, whereas molecular oxygen and reduced NADPH (harbouring one extra electron) are required as co-substrates. H_4B is an essential cofactor for the synthesis of NO because its presence stabilizes the dimeric state of eNOS. L-arginine, H_4B, haem and molecular oxygen bind to the N-terminal oxygenase domain of eNOS, whereas NADPH binds to its C-terminal reductase domain. Under physiological conditions, eNOS catalyses

electron transfer from reduced NADPH of one monomer to the haem-containing oxygenase domain of the other monomer. At this site, oxygen is reduced by the electrons and incorporated into the terminal guanidine group of L-arginine to generate NO and L-citrulline. eNOS exists as a dimer under normal conditions; however, when H_4B is deficient because of oxidative inactivation, the dimer breaks down, resulting in electron transfer to the molecular oxygen to generate superoxide instead of NO[9–11,15,17,22,23,26–29,31,34,35,37,139,140]. This state is referred to as eNOS uncoupling.

H_4B can be generated through two enzymatic pathways: the de novo synthetic pathway and the salvage pathway, which regenerates H_4B from its oxidized form, dihydrobiopterin (H_2B). In the de novo synthesis pathway, H_4B is generated from GTP sequentially by the enzymes GTP cyclohydrolase 1 (GTPCH1; the rate-limiting synthetic enzyme), 6-pyruvoyl tetrahydrobiopterin synthase and sepiapterin reductase (SPR)[24,141]. H_4B can also be regenerated from its oxidized form H_2B in a process catalysed by the rate-limiting, salvage enzyme dihydrofolate reductase (DHFR); H_2B can be converted from the exogenous precursor sepiapterin by SPR.

eNOS uncoupling can occur downstream of NOX activation. Activated NOXs produce ROS, which leads to H_4B deficiency and eNOS uncoupling[15]. The crosstalk and interaction between NOXs and eNOS uncoupling in CVDs is discussed below.

H_4B deficiency-induced eNOS uncoupling has been implicated in various CVDs, including hypertension and aortic aneurysms[13,21,27,29,37], atherosclerosis[18], diabetes mellitus[17,26,142,143], cardiac IR injury[144] and heart failure[16,145]. Specifically, H_4B deficiency and eNOS uncoupling can be induced through DHFR depletion. Knockdown of *Dhfr* leads to eNOS uncoupling[15]. *Dhfr*$^{+/-}$ mice (the homozygous knockout is embryonically lethal) have reduced H_4B levels in the aorta at baseline and a low-level eNOS uncoupling that is well compensated for[37], similar to what is observed in *Apoe*$^{-/-}$ mice[12,29] and *hph-1* mice (a model of GTPCH1 deficiency)[27,32]. However, angiotensin II infusion into *Dhfr*$^{+/-}$ mice resulted in marked hypertension and development of abdominal aortic aneurysm (AAA)[37]. Conversely, upregulation of DHFR recoupled eNOS in animals with hypertension and aortic aneurysms[23,27,36] or diabetes[26], details of which are discussed in the following section.

Additionally, H_4B deficiency and eNOS uncoupling can be caused by deficiency of SPR or GTPCH1, as shown in deoxycorticosterone acetate (DOCA)–salt hypertensive mice[20,21,28,146]. Overexpression of GTPCH1 restored the H_4B level and recoupled eNOS in DOCA–salt hypertensive mice[20,146]. These data indicate that modulation of H_4B metabolic enzymes might be a robust strategy to recouple eNOS as a therapeutic strategy in CVDs.

In addition to H_4B deficiency, other mechanisms have been implicated in inducing eNOS uncoupling[99]. All three isoforms of NOS have a zinc tetrathiolate (ZnS_4) cluster at the dimer interface[147–149]. Oxidants (such as peroxynitrite and hypochlorous acid) disrupt the ZnS_4 cluster of eNOS and result in eNOS uncoupling[150,151]. In addition, *S*-glutathionylation of cysteine residues of eNOS has been shown to induce eNOS uncoupling[152]. In particular, *S*-glutathionylation of aortic eNOS was increased in animal models of hypertension[152], nitrate tolerance[153,154] and streptozotocin (STZ)-induced diabetes[155]. Normalization of *S*-glutathionylation of eNOS in these models reduced eNOS uncoupling and improved vasorelaxation[152–155].

In addition to eNOS uncoupling, uncoupling of nNOS and iNOS has been reported. The first report suggesting that NOS might produce ROS was in the early 1990s, when purified nNOS produced superoxide (then converted to H_2O_2 by superoxide dismutase (SOD)) owing to H_4B or L-arginine deficiency[156,157]. Later, iNOS was shown to catalyse superoxide production under L-arginine-depleted conditions or H_4B deficiency[158,159]. Of note, deficiency of L-arginine is rare under physiological conditions.

Dysfunctional mitochondria

Mitochondria are the cellular energy factory where ATP is synthesized by oxidative phosphorylation[43]. This process relies on a proton gradient generated by the mitochondrial electron transport chain. The electron transport chain comprises a series of complexes that pump protons across the mitochondrial inner

Box 2 | **Development of NOX inhibitors**

1980s
- DPI (1986–1988)[307,308]

1990s
- Apocynin (1990–1992)[309,310]
- AEBSF (1995–1997)[324,325]

2000s
- NOX2ds-tat (2001)[343]
- S17834 (2001)[327]
- HMG-CoA reductase inhibitor (2002)[329,330]
- NSC23766 (2004–2005)[15,334]
- VAS2870 (2006)[336,337]
- Brilliant green and Gentian violet (2006)[340]
- Fulvene-5 (2009)[342]

2010–2012
- GKT136901 and GKT137831 (2010–2012)[347,352,357]
- ML171 (2010–2015)[316,339]
- VAS3947 (2010)[338]
- Imipramine blue (2012)[341]

2013–2015
- NOXA1ds (2013–2018)[132,345]
- 6-(Dimethylamino)fulvene (2014)[60]
- Proton sponge blue (2014)[60]
- GLX351322 (2015)[348]
- GSK2795039 (2015)[350]

2016–2019
- APX-115 (2016–2017)[359,360]
- GLX481372 and GLX7013114 (2018)[349]

AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; apocynin, 4′-hydroxy-3′-methoxyacetophenone; DPI, diphenyleneiodonium; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; NOX, NADPH oxidase; NOXA1, NADPH oxidase activator 1.

membrane to generate a proton gradient, whilst transferring electrons from electron donors (NADH or succinate from the citric acid cycle) to oxygen to generate water. Under normal conditions, electron transportation is efficient and the electron leak is maintained at low, physiological levels[160].

Under conditions of oxidative stress, mitoK$_{ATP}$ is activated by redox-sensitive PKC to transduce redox signals from the cytosol to the mitochondria[39,161,162]. Opening of the mitoK$_{ATP}$ increases the K^+ influx into the mitochondrial matrix, leading to mitochondrial ROS production from the electron transport chain[163–165]. Incubation with 5-hydroxydecanoate, a specific inhibitor of mitoK$_{ATP}$, prevented mitochondrial ROS production, suggesting that K^+ influx has an important role in regulating mitochondrial ROS production[41,166]. At the same time, more electron donors were generated from the citric acid cycle and were pushed into the electron transport chain[167]. Under these conditions, the mitochondrial electron transport chain generates superoxide through electron leakage, when electrons react with oxygen to form superoxide[167,168]. In addition, mitochondrial DNA is damaged by oxidative stress[169], which causes ROS production and apoptosis. The generated superoxide is then rapidly dismutated into H_2O_2 by the mitochondrial isoform of SOD (SOD2), followed by diffusion out of the mitochondria[167,170–172]. Complex I and complex III are reported to be the major sites at which superoxide is generated[164,167,169].

Dysfunctional mitochondria are considered the intracellular source of ROS in various CVDs. Mitochondrial dysfunction has been reported in hypertension[173], atherosclerosis[174], diabetes[175,176], heart failure[177–179] and AAA[37]. The mitochondria-targeted antioxidant MitoQ attenuated cardiac hypertrophy in stroke-prone spontaneously hypertensive rats[173]. MitoQ also reduced ROS production and leukocyte–endothelial cell interactions in leukocytes isolated from patients with diabetes[180]. *Sod2*$^{+/-}$ mice with apolipoprotein E deficiency had greater impairment of vessel relaxation and increased formation of atherosclerotic lesions compared with *Apoe*$^{-/-}$ mice[174,181], implying a critical role of mitochondrial ROS in the development of vascular dysfunction. Overexpression of mitochondrial brown fat uncoupling protein 1 (UCP1) disrupted the mitochondrial electron transport chain and completely inhibited hyperglycaemia-induced mitochondrial superoxide production in mice[182]. Attenuation of mitochondrial ROS by the mitochondria-targeted peptide antioxidant SS-31 preserved insulin sensitivity in rats fed a high-fat diet[176]. In mice, inhibition of mitochondrial ROS production by SS-31 or genetic transfer of catalase targeted to the mitochondria prevented angiotensin II-induced cardiac hypertrophy and diastolic dysfunction[178,183]. Of note, NOX-derived ROS have been shown to enter the mitochondria and promote electron leak and mitochondrial ROS production[4,41], suggesting that dysfunctional mitochondria lie downstream of NOXs.

Xanthine oxidase

Xanthine oxidoreductase is an enzyme initially synthesized in the dehydrogenase form (XDH), which can be rapidly converted into the oxidase form (XO) by oxidation. XDH and XO are interconvertible. Xanthine oxidoreductase is involved in the last two reactions of the purine degradation pathway, converting hypoxanthine to xanthine and then to uric acid[184–186]. In these reactions, XDH favours NAD^+ as the electron acceptor and generates NADH, whereas XO uses oxygen as an electron acceptor and generates superoxide.

Box 3 | Transgenic NOX animal models of CVDs

2000s

- *Nox2*$^{-/y}$ (hypertension) (2001)[56]
- *Ncf1*$^{-/-}$ (atherosclerosis) (2001)[57]
- *Nox2*$^{-/y}$ (heart failure) (2002)[292]
- *Ncf1*$^{-/-}$ (hypertension) (2003)[13]
- *Nox1*$^{-/y}$ (hypertension) (2005–2006)[222,223]
- VSMC-specific *Nox1*-Tg (hypertension) (2005)[235]
- VSMC-specific *Cyba*-Tg (hypertension) (2005)[234]
- EC-specific *Nox2*-Tg (hypertension) (2007)[236]

2010–2012

- *Nox2*$^{-/y}$ (atherosclerosis) (2010)[258]
- *Nox4*$^{-/-}$ (heart failure) (2010)[288]
- Cardiac-specific *Nox4*$^{-/-}$ (heart failure) (2010)[59]
- Cardiac-specific *Nox4*-Tg (heart failure) (2010)[59,288]
- Cardiac-specific *Nox4*-Tg, cardiac-specific *Nox4*-DN-Tg (heart failure) (2010)[89]
- EC-specific *Nox4*-Tg (hypertension) (2011)[241]
- *Nox1*$^{-/y}$ (diabetic vascular function) (2012)[26]
- EC-specific *Nox2*-Tg (atherosclerosis) (2012)[259]
- *Ncf1*$^{-/-}$ (diabetes) (2012)[26]

2013–2015

- *Nox1*$^{-/y}$ (atherosclerosis) (2013)[257]
- *Nox2*$^{-/y}$, *Nox4*$^{-/-}$, cardiac-specific *Nox4*$^{-/-}$, *Nox2*$^{-/y}$ plus cardiac-specific *Nox4*$^{-/-}$ (ischaemia–reperfusion injury) (2013)[58]
- Cardiac-specific *Nox4*-Tg, cardiac-specific *Nox4*-DN-Tg (ischaemia–reperfusion injury) (2013–2014)[58,276]
- *Nox4* or *Nox4*-DN transient overexpression (arrhythmia) (2014)[60]
- VSMC-specific *Cyba*-Tg, VSMC-specific *Cyba*$^{-/-}$ (obesity and diabetes) (2014)[267]
- *Nox4*$^{-/-}$ (atherosclerosis) (2015)[261]
- EC-specific *Nox4*-Tg (atherosclerosis) (2015)[262]
- Podocyte-specific human *NOX5*-Tg (hypertension, diabetic nephropathy) (2014)[248]

2016–2019

- *Nox4*$^{-/-}$ (hypertension) (2016)[249]
- *Ncf1*$^{-/-}$, *Nox1*$^{-/y}$, *Nox2*$^{-/y}$, *Nox4*$^{-/-}$ (abdominal aortic aneurysm) (2017)[34]
- VSMC-specific human *NOX5*-Tg (diabetic nephropathy) (2017)[246]
- *Nox4*$^{-/-}$ (hypertension) (2018)[238]
- VSMC-specific human *NOX5*-Tg (vasorelaxation) (2018)[245]
- EC-specific human *NOX5*-knock-in (stroke) (2019)[247]

CVD, cardiovascular disease; DN, dominant negative; EC, endothelial cell; NOX, NADPH oxidase; Tg, transgenic; VSMC, vascular smooth muscle cell.

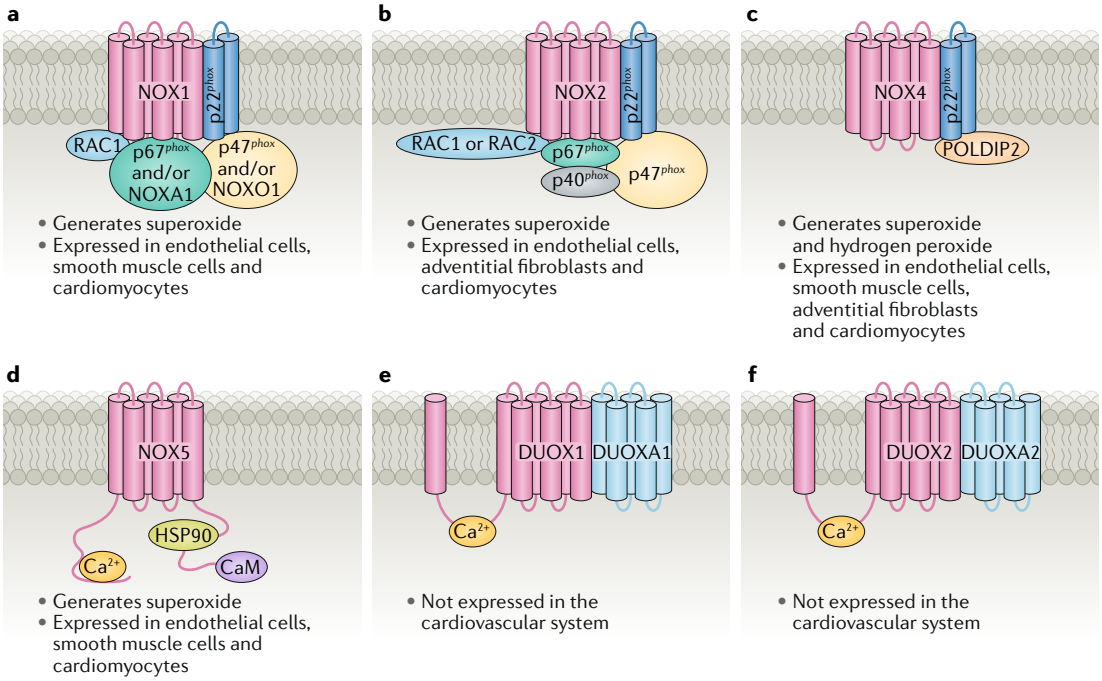

Fig. 2 | **Composition and cell-specific expression and activity of NOX isoforms in the cardiovascular system.** **a** | NADPH oxidase 1 (NOX1). **b** | NOX2. **c** | NOX4. **d** | NOX5. **e** | Dual oxidase 1 (DUOX1). **f** | DUOX2. CaM, calmodulin; DUOXA, dual oxidase maturation factor; HSP90, heat shock protein 90; NOXA1, NADPH oxidase activator 1; NOXO1, NADPH oxidase organizer 1; POLDIP2, polymerase δ-interacting protein 2.

Studies suggest that XO is involved in the progression of various CVDs. The administration of an XO inhibitor was beneficial in animal models of hypertension[187–189], myocardial IR injury[190,191] and chronic heart failure[192,193]. However, use of an XO inhibitor (300–600 mg per day) did not show benefits in patients with hypertension or chronic heart failure[194–196]. A retrospective analysis in patients with hyperuricaemia and acute MI suggested that the combination of an XO inhibitor and an angiotensin-converting enzyme (ACE) inhibitor protected against major cardiovascular events (death or hospitalization for cardiovascular causes) after acute MI compared with treatment with an ACE inhibitor alone[197]. These data suggest that XO inhibition might have limited beneficial effects only in patients with hyperuricaemia and CVDs. Given that inhibition of NOX activity suppresses XO activation and superoxide production[198], the roles of NOX–XO crosstalk in the pathogenesis of CVDs are discussed below.

Cardiovascular oxidase crosstalk

NOXs have been shown to be the primary oxidases activated in the cardiovascular system, but accumulating data indicate that complex crosstalk exists between NOXs and other ROS-generating enzymes or enzymatic systems, including uncoupled eNOS, dysfunctional mitochondria and XO. These secondary oxidase systems can also activate NOXs and/or each other. The interactions between these oxidases in the cardiovascular system are introduced in this section; the contributions of oxidase crosstalk to particular CVDs are then discussed in detail in the next section.

NOXs and uncoupled eNOS

Transient exposure (30 min) of bovine endothelial cells to angiotensin II in vitro increased the production of superoxide, which was attenuated by the RAC1 inhibitor NSC23766, indicating NOX-derived ROS production[15]. However, after 24 h of angiotensin II treatment, superoxide production was completely blocked by administration of L-NAME (a NOS inhibitor), whereas NSC23766 did not significantly reduce superoxide production[15]. These data suggest that uncoupled eNOS is predominantly responsible for ROS production after prolonged exposure of endothelial cells to angiotensin II, and that eNOS uncoupling occurs as a consequence of angiotensin II-induced activation of NOX[15].

NOX activation induces uncoupling of eNOS through E2F1-dependent, E2F2-dependent or E2F3a-dependent downregulation of *Dhfr* expression[15,36]. In bovine endothelial cells, angiotensin II-induced NOX activation leads to H_2O_2 production[15]. In turn, H_2O_2 downregulated the expression of E2F1, E2F2 and E2F3a, the main transcription factors required to activate *Dhfr* transcription in endothelial cells[36]. As a result, the expression and activity of DHFR were attenuated, leading to persistent H_4B deficiency and eNOS uncoupling[15,36]. In mouse models, angiotensin II infusion induces endothelial DHFR deficiency and eNOS uncoupling[27,29,32]. Restoration of endothelial DHFR expression and activity with oral folic acid administration or in vivo transfection of *Dhfr* recoupled eNOS and improved NO bioavailability in angiotensin II-infused animals, resulting in lowered blood pressure[15,27,29]. Similarly, adenovirus-delivered E2F1 overexpression in mice significantly increased DHFR protein abundance and H_4B

bioavailability and recoupled eNOS[36]. NO bioavailability was also restored, resulting in reduced blood pressure[36]. These data reveal a novel pathway of NOX–H_2O_2–E2F–DHFR-dependent regulation of eNOS uncoupling and its role in elevating blood pressure.

In addition, eNOS uncoupling develops in DOCA–salt hypertensive mice and rats and is associated with H_4B deficiency[13,20,28,199]. This deficiency has been shown to result from decreased SPR expression and GTPCH1 activity, both of which lead to impaired H_4B bioavailability[86,88]. NOX activity was also reported to be upregulated in DOCA–salt hypertensive mice[200]. Application of the NOX inhibitor 4'-hydroxy-3'-methoxyacetophenone (apocynin; later shown to be a nonspecific inhibitor of all flavin-containing enzymes, including NOXs) or deletion of $p47^{phox}$ restored H_4B bioavailability and eNOS coupling in DOCA–salt hypertensive mice[13,28]. These data demonstrate an upstream role of NOXs in eNOS uncoupling in a salt-sensitive model of hypertension. Of note, different H_4B metabolic enzymes are involved in different types of hypertension, with DHFR deficiency underlying eNOS uncoupling and hypertension in angiotensin II-infused mice, and SPR and GTPCH1 deficiency accounting for hypertension in DOCA–salt hypertensive animals.

The interaction between NOXs and uncoupled eNOS has also been studied in STZ-injected animals. STZ injection in mice downregulated aortic DHFR expression and H_4B bioavailability, resulting in eNOS uncoupling[17,26]. Attenuation of angiotensin II signalling in STZ-injected mice by oral administration of the angiotensin II receptor type 1 (AT_1) blocker candesartan or the ACE inhibitor captopril recoupled eNOS through inhibition of NOX activity and restoration of DHFR protein expression[17]. Further investigation demonstrated that knockout of either *Nox1* or *Ncf1* (encoding $p47^{phox}$), or in vivo knockdown of *Noxo1* by RNA interference, improved endothelium-dependent vasodilatation in STZ-induced diabetic mice[26]. This improvement was attributed to recoupling of eNOS as a result of the restoration of DHFR function and H_4B bioavailability[26]. These data strongly implicate a selective role of NOX1 in activating eNOS uncoupling via angiotensin II signalling in STZ-injected type 1 diabetic mice. By contrast, in *db*/*db* type 2 diabetic mice, infusion of the bone morphogenetic protein 4 (BMP4) antagonist noggin attenuated eNOS uncoupling through inhibition of NOX1 (REF.[35]). Together, these results strongly indicate NOX-dependent uncoupling of eNOS through NOX-derived ROS production and oxidation of H_4B.

NOXs and mitochondria

Angiotensin II-induced NOX activation has been reported to induce mitochondrial ROS production and mitochondrial dysfunction in endothelial cells[41,201,202]. Inhibition of NOX activity with apocynin or with small interfering RNA (siRNA) targeted to *Cyba* (encoding $p22^{phox}$) in bovine endothelial cells in vitro reduced angiotensin II-provoked mitochondrial ROS production, indicating NOX-dependent modulation of mitochondrial dysfunction[41,202]. This modulation seems to be mediated by uncoupling of eNOS. Treatment with the NOS inhibitor L-NAME prevented angiotensin II-induced mitochondrial dysfunction[41]. Angiotensin II-stimulated mitochondrial ROS production is also reported to involve the opening of $mitoK_{ATP}$ in both endothelial cells and vascular smooth muscle cells[41,166].

Conversely, feedback regulation of mitochondria on NOXs has also been reported. Opening of $mitoK_{ATP}$ by treatment with diazoxide results in NOX activation[166]. Moreover, treatment with the $mitoK_{ATP}$-specific inhibitor 5-hydroxydecanoate reduced superoxide production (generated by NOXs and uncoupled eNOS) in angiotensin II-treated endothelial cells in vitro, suggesting feedback regulation of NOX and eNOS activity by $mitoK_{ATP}$ (REFS[38,39,41]). As discussed above, PKC and Src induce NOX activation through $p47^{phox}$ phosphorylation and the EGFR–PI3K–RAC1 axis, respectively[39,97–99,101]. In mice, direct clearance of mitochondrial superoxide by either overexpression of SOD2 or the administration of the mitochondria-targeted antioxidant MitoTEMPO inhibited NOX activity in endothelial cells[203]. Of note, SOD2 or MitoTEMPO had no effects on basal NOX activity and inhibited NOX activation only in angiotensin II-stimulated cells[39,203].

Uncoupled eNOS and mitochondria

An interaction between uncoupled eNOS and mitochondria has been reported in endothelial cells[41]. Superoxide reacts with NO to form peroxynitrite, which can damage mitochondria through oxidation of membrane lipids and electron transport chain complexes[204,205]. Administration of uric acid, a scavenger of peroxynitrite, or L-NAME protected against angiotensin II-induced mitochondrial dysfunction in cultured endothelial cells, indicating eNOS-dependent mitochondrial dysfunction[41]. Angiotensin II-infused $Dhfr^{+/-}$ mice have dramatically increased mitochondrial superoxide production in the aorta, suggesting that DHFR deficiency-dependent eNOS uncoupling induces mitochondrial dysfunction[37]. An upstream role of uncoupled eNOS in mediating mitochondrial dysfunction has also been reported in the heart[206]. Uncoupling of eNOS induced by treatment of mice with 2,4-diamino-6-hydroxypyrimidine (DAHP; an inhibitor of GTPCH1) resulted in H_4B depletion, impaired mitochondrial function in the heart, and cardiac contractile dysfunction[206]. In mice with cardiac IR injury, treatment with sepiapterin (a precursor of H_4B) recoupled eNOS to reduce mitochondrial superoxide production, resulting in preserved cardiac mitochondrial function and cardiac function[31].

Conversely, mitochondrial ROS production might also regulate eNOS coupling–uncoupling activity. In humans, restoration of mitochondrial electron transport by supplementation with antioxidant coenzyme Q10 recoupled eNOS and resulted in improved endothelial function in diabetes and atherosclerosis[207,208]. Additionally, inhibition of $mitoK_{ATP}$ by 5-hydroxydecanoate completely restored NO production in angiotensin II-treated endothelial cells[41]. Although eNOS uncoupling was not directly measured in this study, restored NO production indicated improved eNOS function and a reduced uncoupling status[41].

NOXs, XO and mitochondria

Apocynin treatment reportedly prevented XO activation and superoxide production in IR-injured guinea pig hearts[198]. However, inhibition of XO by allopurinol or tungsten did not modulate NOX activity[198], suggesting that XO acts downstream of NOX in IR injury.

An interaction between XO and mitochondria has been reported in vivo. In a rat model of cocaine-induced cardiac dysfunction, treatment with allopurinol significantly reduced mitochondrial ROS production and improved cardiac function[209]. In left ventricular cardiomyocytes isolated from adult rats, application of the mitochondrial inhibitor MitoQ prevented stretch-induced XO activation, indicating a self-perpetuating cycle between XO and mitochondria[210]. The mechanisms might involve hypoxanthine, a metabolic product of ADP and AMP, both of which are produced by the breakdown of ATP from mitochondria[210]. Hypoxanthine reacts with XO to produce superoxide, which in turn causes damage to mitochondria[210].

Oxidase crosstalk in CVDs

Emerging evidence indicates that oxidase crosstalk is a major mechanism underlying sustained oxidative stress during cardiovascular pathogenesis. The primary oxidase system that is first activated seems to be NOXs, which can activate downstream oxidases or oxidase systems, such as uncoupled eNOS, dysfunctional mitochondria or XO, resulting in secondary production of ROS. The detailed molecular pathways and pathophysiological relevance of the oxidase crosstalk in CVDs, including hypertension, AAA, hypercholesterolaemia, atherosclerosis, diabetic vascular dysfunction, cardiac IR injury, heart failure and cardiac arrhythmias, are discussed below. The investigations of animal models with genetic modifications of various NOX isoforms and subunits discussed in this section are summarized in TABLE 1.

Hypertension

Increased vascular ROS production in hypertension has long been reported in animal models treated with angiotensin II[211,212], DOCA–salt[28,213,214] or L-NAME[215]. The association between elevated vascular ROS production and hypertension has also been reported in animals with genetic modifications or in inbred strains, including Dahl salt-sensitive rats[216] and spontaneously hypertensive rats[217]. NOX and uncoupled eNOS have important roles in the elevation of blood pressure[23,27,36,218–220]. Angiotensin II is a potent vasoconstrictive peptide that induces hypertension through the activation of vascular NOX and NOX-derived ROS[15,23,27,32,37,211]. Specifically, increased levels of *Nox1*, *Nox2* and *Nox4* mRNA were reported in aortas from angiotensin II-infused animals[221,222]. In vitro, angiotensin II has been shown to upregulate *Nox1* mRNA and NOX1 protein levels as well as *Nox4* mRNA levels in vascular smooth muscle cells[223,224] and to upregulate both NOX2 and NOX4 protein levels and activity in endothelial cells[225,226]. Expression of NOX5 was also found to be upregulated by angiotensin II in human cultured endothelial cells[227]. Previous studies have demonstrated that angiotensin II activates NOX via AT_1-dependent phosphorylation of $p47^{phox}$ through a signalling pathway involving Src, PKC and phospholipase D[101,228,229]. Conversely, Src-induced EGFR transactivation and PI3K activation lead to the activation of RAC1, an essential event in the activation of NOX[101]. The detailed mechanisms of activation of NOX by angiotensin II have been previously reviewed[5,230–232].

Infusion of angiotensin II increases aortic superoxide production and blood pressure in wild-type mice[212,222,233]. Overexpression of $p22^{phox}$ in vascular smooth muscle cells exacerbated angiotensin II-induced hypertension in mice[234]. Overexpression of NOX1 in vascular smooth muscle further increased angiotensin II-induced aortic superoxide production and hypertension in mice, both of which were corrected by administration of the antioxidant Tempol[235]. Deletion of *Nox1* attenuated oxidative stress and hypertension in angiotensin II-infused mice[222,233]. Given that NOX1 was shown to be upregulated by angiotensin II only in vascular smooth muscle cells, and not in endothelial cells[223,224], vascular smooth muscle NOX1 might have a more important role than endothelial NOX1 in the development of hypertension. Moreover, knockout of *Nox2* in mice attenuated aortic superoxide production and blood-pressure elevation in response to angiotensin II[56]. Endothelium-specific overexpression of NOX2 in mice exacerbated the angiotensin II-induced increase in blood pressure[236,237]. Although NOX2 was not reported to be regulated by angiotensin II in vascular smooth muscle cells, endothelial NOX2 might, however, be involved in the regulation of angiotensin II-induced hypertension.

The role of NOX4 in hypertension has been studied, with inconsistent results. $Nox4^{-/-}$ mice had a lower blood pressure increase in response to angiotensin II infusion than wild-type mice[238]. However, another study showed that inducible deletion of *Nox4* had no effect on basal blood pressure or angiotensin II-induced hypertension (conditional *Nox4* deletion after initiation of angiotensin II infusion)[239]. These results suggest that NOX4 might be involved in the initiation rather than the maintenance of angiotensin II-induced hypertension in mice. Interestingly, cardiac-specific overexpression of NOX4 did not modulate blood pressure in angiotensin II-infused mice[240], suggesting that vascular rather than cardiac NOX4 has an important role in the regulation of blood pressure. Contrary to the results in $Nox4^{-/-}$ mice, endothelium-specific overexpression of NOX4 in mice decreased the angiotensin II-induced rise in blood pressure owing to increased H_2O_2 production and endothelium-dependent vasorelaxation[241]. Perhaps these results can be interpreted as a compensatory response of H_2O_2-dependent vasodilatation on blood pressure[242–244].

Rodents do not have NOX5. In transgenic mice with vascular smooth muscle-specific expression of human *NOX5*, baseline blood pressure levels and the angiotensin II-induced elevation in blood pressure were not different from levels in wild-type mice[245,246]. Given that angiotensin II induces the upregulation of NOX5 expression and activity in human cultured endothelial cells, investigation of endothelial NOX5 in angiotensin II-dependent hypertension in patients is important[227].

Table 1 | **NADPH oxidase genetically modified animal models**

Gene	Type of modification	Genetic modification	Disease model	Experimental results[a]	Refs
Nox1	Global knockout	*Nox1*$^{-/y}$	ANGII infusion	Attenuated ANGII-induced hypertension	222,233
		Nox1$^{-/y}$ on *hph-1* background[233]	ANGII infusion	Prevented eNOS uncoupling in the aorta and reduced incidence of AAA	34
		Nox1$^{-/y}$ on *Apoe*$^{-/-}$ background[233]	High-fat diet or STZ injection	Reduced superoxide production in the aorta and reduced aortic lesion formation	256,257
		Nox1$^{-/y}$ (REF.[233])	STZ injection	Inhibited aortic eNOS uncoupling and improved endothelium-dependent vasorelaxation	26
	Vascular smooth muscle transgene	Overexpression of human *NOX1* in vascular smooth muscle cells	ANGII infusion	Increased aortic superoxide production and exaggerated increase in BP in ANGII-treated animals	235
Nox2 (*Cybb*)	Global knockout	*Nox2*$^{-/y}$	ANGII infusion	Inhibited ANGII-induced superoxide production in the aorta and reduction or no change in BP at baseline or in response to ANGII infusion	56,372
		Nox2$^{-/y}$ on *hph-1* background	ANGII infusion	Prevented eNOS uncoupling in the aorta and reduced incidence of AAA	34
		Nox2$^{-/y}$ on *Apoe*$^{-/-}$ background[373]	High-fat diet	Reduced aortic superoxide production and reduced aortic lesion formation	258
		Nox2$^{-/y}$	Cardiac IR injury	Reduced cardiac superoxide production, attenuated cardiomyocyte apoptosis and reduced IR injury in the heart	58
		Nox2$^{-/y}$ plus cardiac-specific *Nox4*$^{-/-}$	Cardiac IR injury	Reduced superoxide production, increased cardiomyocyte apoptosis and increased IR injury in the heart	58
		Nox2$^{-/y}$	ANGII infusion or TAC	Inhibited ANGII-induced myocardial NOX activity or superoxide production, reduced cardiac fibrosis in ANGII-infused animals, and no change or attenuated cardiac hypertrophy	292,294,372
	Endothelial transgene	Overexpression of human *NOX2* in endothelial cells	ANGII infusion	Increased NOX activity or superoxide production, increased BP in ANGII-infused animals (0.3–0.4 mg/kg per day) and no change in ANGII-induced hypertension (1.1 mg/kg per day)	236,237,372
		Overexpression of human *NOX2* in endothelial cells on *Apoe*$^{-/-}$ background[236]	ANGII infusion	Increased endothelial superoxide production at baseline and no change in atherosclerotic lesion formation	259
		Overexpression of human *NOX2* in endothelial cells[237]	ANGII infusion or left coronary artery ligation	No change in hypertrophy or infarct area	275,372
	Cardiac transgene	Overexpression of human NOX2 in cardiomyocytes	Left coronary artery ligation	No change in infarct area and same mortality	275
Nox4	Global knockout	*Nox4*$^{-/-}$	ANGII infusion	No change or decreased BP in response to ANGII infusion	238,239
		Nox4$^{-/-}$ in Dahl salt-sensitive rats	4.0% NaCl diet	Reduced kidney oxidative stress and attenuated BP in salt diet-treated animals	249
		Nox4$^{-/-}$ on *hph-1* background	ANGII infusion	Prevented eNOS uncoupling in the aorta and reduced incidence of AAA	34
		Nox4$^{-/-}$ on *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ background	High-fat diet with or without partial carotid artery ligation, or STZ injection	Increased aortic superoxide production and increased atherosclerotic lesion formation	243,260,261
		Nox4$^{-/-}$	Cardiac IR injury	Reduced cardiac superoxide production, inhibited cardiomyocyte apoptosis and protected from IR injury in the heart	58
		Nox4$^{-/-}$	Suprarenal aortic constriction or TAC	Exaggerated cardiac fibrosis and increased cardiac hypertrophy	134,288
		Knockdown of *Nox4* by in vivo siRNA injection	Cardiac IR injury (Langendorff)	Recoupled eNOS in IR-injured heart, attenuated mitochondrial superoxide production and reduced cardiac IR injury	31

Table 1 (cont.) | **NADPH oxidase genetically modified animal models**

Gene	Type of modification	Genetic modification	Disease model	Experimental results[a]	Refs
Nox4 (cont.)	Cardiac knockout	Cardiac-specific *Nox4*$^{-/-}$	Cardiac IR injury	Reduced cardiac superoxide production, inhibited cardiomyocyte apoptosis and protected from IR injury in the heart	58
		Cardiac-specific *Nox4*$^{-/-}$	Phenylephrine infusion or TAC	Reduced cardiac hypertrophy	59,134
	Global transient overexpression	Overexpression of human *NOX4*	NA	Induced arrhythmic phenotype in zebrafish embryos	60
		Overexpression of dominant-negative form of human *NOX4* (P437H)	NA	Abrogated arrhythmic phenotype in zebrafish embryos	60
	Endothelial transgene	Overexpression of *Nox4* in endothelial cells	ANGII infusion	Elevated H_2O_2 production in endothelial cells, improved endothelial-dependent vasodilatation and reduced BP in ANGII-infused animals	241
		Overexpression of *Nox4* in endothelial cells on *Apoe*$^{-/-}$ background	High-fat and high-cholesterol diet	Attenuated atherosclerosis	262
	Cardiac transgene	Inducible overexpression of *Nox4* in cardiomyocytes	ANGII infusion	No change in mean BP in response to ANGII infusion	240
		Overexpression of *Nox4* in cardiomyocytes[89]	Cardiac IR injury (in vivo or Langendorff)	Increased ROS production and no alteration or exacerbation in cardiac IR injury	58,276
		Overexpression of dominant-negative form of *Nox4* (P437H) in cardiomyocytes[89]	Cardiac IR injury (in vivo or Langendorff)	Increased superoxide production and increased IR injury in the heart	58,276
		Overexpression of *Nox4* in cardiomyocytes	Ageing, TAC or phenylephrine or ANGII infusion	Increased ROS production and apoptosis, diminished left ventricle function and increased hypertrophy (at cellular level of cardiomyocytes and at the organ level)	59,89, 134,240
		Overexpression of *Nox4* in cardiomyocytes	Suprarenal aortic constriction	Increased H_2O_2 production in the heart at baseline, increased myocardial angiogenesis, protected from cardiac dysfunction and fibrosis, and reduced cardiac hypertrophy	288
		Overexpression of dominant-negative form of *Nox4* (P437H) in cardiomyocytes[89]	Ageing	Decreased superoxide production in left ventricle and no change in cardiac fibrosis or apoptosis	89
Nox5	Endothelial knock-in	Knock-in of human *NOX5* in endothelial cells	Cardiac IR injury, MI or brain IR injury (stroke–reperfusion)	No change in BP at baseline, no change in cardiac infarct size or cardiac function after cardiac IR injury or MI, increased ROS production after stroke, increased blood–brain barrier leakage and increased infarct size after brain IR injury	247
	Vascular smooth muscle overexpression	Overexpression of human *NOX5* in vascular smooth muscle cells	STZ injection	Increased ROS production and increased diabetic nephropathy	246
	Vascular smooth muscle overexpression	Overexpression of human *NOX5* in vascular smooth muscle cells	ANGII infusion	Increased ROS in the vessels and the heart, impaired endothelium-dependent vasorelaxation and no change in BP at baseline or in response to ANGII infusion	245
	Podocyte overexpression	Overexpression of human *NOX5* in podocytes	STZ injection	Increased ROS production, increased BP at baseline plus further increase in response to STZ and increased renal damage in response to STZ	248
Cyba (encoding $p22^{phox}$)	Vascular smooth muscle knockout	Smooth muscle-specific *Cyba*$^{-/-}$	High-fat diet	Reduced perivascular inflammation	267
	Vascular smooth muscle overexpression	Overexpression of *Cyba* in vascular smooth muscle cells	ANGII infusion	Increased aortic H_2O_2 production and exaggerated BP increase in ANGII-treated animals	234
		Overexpression of *Cyba* in vascular smooth muscle cells	High-fat diet	Increased skeletal mitochondrial superoxide production and impaired skeletal mitochondrial function	267

Table 1 (cont.) | **NADPH oxidase genetically modified animal models**

Gene	Type of modification	Genetic modification	Disease model	Experimental results[a]	Refs
Ncf1 (encoding p47^{phox})	Global knockout	*Ncf1*$^{-/-}$ (REF. [374])	DOCA–salt, 1% saline, left kidney removal	Increased aortic H_4B bioavailability, reduced aortic superoxide and H_2O_2 production, and reduced BP	13
		Ncf1$^{-/-}$ on *hph-1* background	ANGII infusion	Prevented eNOS uncoupling in the aorta and reduced incidence of AAA	34
		Ncf1$^{-/-}$ on *Apoe*$^{-/-}$ background	High-fat, atherogenic diet	Reduced aortic ROS production and decreased lesion formation	57
		Ncf1$^{-/-}$	STZ injection	Recoupling of eNOS in STZ-injected animals	26
Noxo1	Global knockdown	Knockdown of *Noxo1* by in vivo siRNA injection	STZ injection	Recoupling of eNOS in STZ-injected animals	26

AAA, abdominal aortic aneurysm; ANGII, angiotensin II; BP, blood pressure; DOCA, deoxycorticosterone acetate; eNOS, endothelial nitric oxide synthase; H_2O_2, hydrogen peroxide; IR, ischaemia–reperfusion; MI, myocardial infarction; NA, not applicable; NOX, NADPH oxidase; ROS, reactive oxygen species; siRNA, small interfering RNA; STZ, streptozotocin; TAC, transverse aortic constriction. [a]Compared with nongenetically modified animals on the same genetic background and with the same treatment.

Basal blood pressure levels in mice with endothelial knock-in of human *NOX5* with the use of a *Tie2* promoter was not different from that in wild-type animals[247]. Interestingly, transgenic mice expressing human *NOX5* specifically in podocytes had elevated blood pressure, which was further exacerbated by STZ-induced diabetes owing to severe renal damage[248]. Taken together, these data suggest that activation of NOXs has an important role in the development of angiotensin II-induced hypertension in animal models. Global knockout of either *Nox1*, *Nox2* or *Nox4* protected against angiotensin II-induced hypertension[56,222,233,238], implicating an upstream role of NOX1, NOX2 and/or NOX4 in the development of this type of hypertension.

In addition to angiotensin II-dependent hypertension, NOXs have been shown to be involved in the regulation of blood pressure in other models of hypertension. In mice and rats with DOCA–salt hypertension, aortic expression of p22^{phox} and superoxide production were both increased[28,213]. Treatment with apocynin significantly reduced superoxide production and decreased blood pressure in these animals[28,213]. Global deletion of *Ncf1* abrogated aortic superoxide production and hypertension in DOCA–salt mice[13]. In addition, deletion of *Nox4* has been shown to attenuate renal oxidative stress and hypertension in Dahl salt-sensitive rats[249].

As discussed above, NOX-dependent ROS production leads to eNOS uncoupling. In vivo evidence also supports the upstream role of NOX in inducing eNOS uncoupling in the development of hypertension[222,233]. Deletion of *Nox1* protected against vascular dysfunction and hypertension in response to angiotensin II infusion in mice[34,37,222,233]. Interestingly, administration of L-NAME diminished the protective effects of *Nox1* deletion[222], strongly indicating an intermediate role of eNOS uncoupling in the NOX1-triggered development of angiotensin II-induced hypertension. Moreover, recoupling of eNOS has been reported to attenuate hypertension in angiotensin II-infused or DOCA–salt-treated mice[13,20,27,33,36]. Adenovirus-mediated overexpression of E2F1 led to eNOS recoupling and normalized blood pressure in angiotensin II-infused mice[36]. Endothelial overexpression of GTPCH1 improved H_4B bioavailability, recoupled eNOS and reduced blood pressure in DOCA–salt-treated mice[20]. Direct supplementation of H_4B recoupled eNOS and reduced blood pressure in DOCA–salt mice[13]. *hph-1* mice have a mutation in *Gch1* (encoding GTPCH1) and have a phenotype of modest eNOS uncoupling that is well compensated for by H_2O_2-dependent vasodilatation[27]. Blood pressure in these mice is elevated by only 10 mmHg at baseline compared with wild-type mice[27]. With angiotensin II infusion, eNOS uncoupling was tripled in *hph-1* mice, which resulted in severe vascular remodelling and the formation of AAA[27]. Oral administration of folic acid to restore endothelial DHFR function and recouple eNOS normalized blood pressure in angiotensin II-infused wild-type mice and prevented aneurysm-related blood pressure decline in *hph-1* mice[27]. These findings suggest that NOX-dependent eNOS uncoupling has an important role in the development of hypertension and AAA (discussed further below).

Dhfr$^{+/-}$ mice have an exaggerated elevation in blood pressure in response to angiotensin II infusion owing to exacerbated eNOS uncoupling activity and mitochondrial dysfunction[37]. Administration of MitoTEMPO in these mice significantly reduced angiotensin II-induced high blood pressure and AAA formation[37], implicating eNOS uncoupling-dependent mitochondrial dysfunction in the development of hypertension and AAA.

In SOD2-deficient mice, angiotensin II-induced NOX activation, eNOS uncoupling and high blood pressure were further elevated compared with wild-type animals receiving the same treatment, suggesting crosstalk between mitochondrial ROS, NOX activation and eNOS uncoupling[250]. Importantly, angiotensin II-induced NOX activation, eNOS uncoupling and hypertension can be blocked by inhibition of the mitochondrial membrane permeability transition pore (by cyclophilin D deficiency or sanglifehrin A treatment), implying an essential role of mitochondrial ROS in the modulation of NOX activity and eNOS uncoupling in angiotensin II-induced hypertension[250].

Aortic aneurysms

Whereas a certain degree of eNOS uncoupling mediates hypertension, more extensive eNOS uncoupling induces the formation of AAA. As mentioned above, angiotensin II-infused *hph-1* mice had a threefold increase in eNOS uncoupling, accompanied by a 79% incidence

of AAA and a 14% rate of AAA rupture within 2 weeks[27]. This mouse is a novel and robust model of AAA. The traditional models of AAA, such as angiotensin II-infused *Apoe*$^{-/-}$ mice, usually take 4 weeks to develop AAA, and the aneurysms rarely rupture. Administration of folic acid, which restores endothelial DHFR expression and activity to recouple eNOS, completely prevented the development of AAA in angiotensin II-infused *hph-1* mice[27], indicating a causal role of uncoupled eNOS in the formation of AAA. In addition, eNOS uncoupling mediates AAA formation in the angiotensin II-infused *Apoe*$^{-/-}$ mouse model of AAA[29]. Similar to *hph-1* mice, *Apoe*$^{-/-}$ mice have minimal eNOS uncoupling activity at baseline and a compensated phenotype of normal vasodilatation[27,29]. With angiotensin II infusion, aortic eNOS uncoupling was markedly increased in these mice, accompanied by severe vascular remodelling and the development of AAA (92% incidence)[29]. Recoupling of eNOS by restoration of endothelial DHFR function through folic acid supplementation substantially reduced angiotensin II-induced AAA formation to 22%[29]. In *Dhfr*$^{+/-}$ mice, angiotensin II infusion also resulted in a significantly higher incidence of AAA compared with wild-type littermates with the same genetic background[37]. These findings strongly indicate a central causal role of eNOS uncoupling in the development of AAA. Furthermore, we have established in mice that circulating H_4B levels can be used as a novel and powerful biomarker for AAA development and response to treatment[30]. Circulating levels of H_4B are accurately and linearly correlated with aortic H_4B levels in angiotensin II-treated *hph-1* mice and *Apoe*$^{-/-}$ mice[30]. Reduced circulating H_4B levels are associated with an increased incidence of AAA, whereas prevention of AAA with folic acid dietary supplementation is associated with fully restored circulating H_4B levels[30].

As an upstream activator of uncoupled eNOS, the NOX family has been studied for their roles in the formation of AAA. NOX activity was upregulated in aortic tissues from AAA in patients[251]. NOX inhibitors (diphenyliodonium and apocynin) potently reduced superoxide production in patients with AAA, indicating an important role of NOXs in AAA formation[252,253]. Expression of $p22^{phox}$, $p47^{phox}$, NOX2 and NOX5 was found to be upregulated in AAA in patients[251,252]. Two novel *NOX4* mutations were identified in patients with AAA[34]. These mutations are associated with a markedly increased H_2O_2 production[34]. In *hph-1* mice, deletion of *Nox1*, *Nox2*, *Nox4* or *Ncf1* was sufficient to prevent AAA formation with angiotensin II infusion[34]. Consistent with our previous findings of a critical role of eNOS uncoupling in AAA formation in angiotensin II-infused *hph-1* mice[27], deletion of *Nox1*, *Nox2*, *Nox4* or *Ncf1* on the *hph-1* background restored endothelial DHFR function and recoupled eNOS to attenuate AAA formation[34]. These data establish an essential role of NOX1-dependent, NOX2-dependent and/or NOX4-dependent eNOS uncoupling in the development of AAA. Recoupling of eNOS by targeting DHFR deficiency or NOX1, NOX2, or NOX4 might be a novel therapeutic approach for the prevention and treatment of AAA.

Our findings in *Dhfr*$^{+/-}$ mice have shown that mitochondria act downstream of eNOS uncoupling in modulating the development of AAA[37]. Application of MitoTEMPO completely blocked the development of AAA in angiotensin II-infused *Dhfr*$^{+/-}$ mice[37]. These data indicate that eNOS uncoupling induces AAA formation through mitochondrial dysfunction, targeting of which might be a novel and effective therapeutic strategy for AAA. Consistent with the notion that eNOS uncoupling has a causal role in AAA formation, we have shown that two doses of nifedipine (an L-type Ca^{2+}-channel blocker) treatment prevented AAA formation in angiotensin II-infused *hph-1* mice via inhibition of NOX activity and eNOS uncoupling[32]. Whereas a low dose of nifedipine is ineffective in reducing blood pressure, a high dose of nifedipine is effective in reducing both blood pressure and the formation of AAA in mice. These data indicate that nifedipine might be a particularly useful treatment for patients with coexisting hypertension and AAA.

Hypercholesterolaemia and atherosclerosis

NOX-derived oxidative stress has been shown to be a major mediator of atherosclerosis[254]. LDL oxidation, a major event during early atherogenesis, can be induced by NOX-derived ROS[255]. Roles of NOXs in atherosclerosis have been investigated in genetically modified animal models on the background of *Apoe*$^{-/-}$, a widely used model of atherogenesis. In *Apoe*$^{-/-}$ mice, knockout of *Ncf1* protected against lesion formation, which suggests that either NOX1 or NOX2 or both are required for the development of atherosclerosis[57]. Specifically, deletion of *Nox1* in *Apoe*$^{-/-}$ mice reduced aortic superoxide production, macrophage infiltration and lesion formation[256,257]. Administration of GKT137831, an inhibitor of NOX1 and NOX4, had similar effects to those of *Nox1* deletion in *Apoe*$^{-/-}$ mice[257]. Likewise, deletion of *Nox2* in *Apoe*$^{-/-}$ mice resulted in decreased aortic superoxide production, reduced lesion formation and increased NO bioavailability[258]. Of note, endothelium-specific overexpression of NOX2 did not further accelerate the progression of atherosclerosis in *Apoe*$^{-/-}$ mice, although superoxide production and macrophage recruitment were increased[259]. These results indicate that NOX1 and NOX2 have critical roles in atherogenesis, and cell type-specific contributions of NOXs and NOX-derived ROS warrant further investigation.

Several studies have produced evidence to support a protective role of NOX4 in atherosclerosis[243,260–262]. Global *Nox4* knockout or induced deletion of *Nox4* increased atherosclerosis in *Apoe*$^{-/-}$ mice[260,261]. Although H_2O_2 production was reduced, increased inflammation, macrophage accumulation and fibrosis were noted in the aortas of *Nox4*$^{-/-}$*Apoe*$^{-/-}$ mice[260,261]. The researchers concluded that NOX4-produced H_2O_2 has anti-atherosclerotic functions[260,261]. In accordance with these results, endothelial overexpression of NOX4 protected *Apoe*$^{-/-}$ mice from the formation of atherosclerotic lesions, primarily through attenuated inflammatory responses[262]. In *Ldlr*$^{-/-}$ mice, deletion of *Nox4* resulted in increased atherosclerotic lesion formation mediated by H_2O_2 deficiency and endothelial dysfunction[243]. Together, these results suggest that NOX4-derived H_2O_2

might mediate beneficial effects in atherosclerosis via inhibition of inflammation, which is contrary to the deleterious effects of ROS produced by NOX1 and NOX2. These findings in mouse models are in agreement with the findings that *NOX4* mRNA levels were decreased whereas *NOX1* mRNA levels were increased in endarterectomy specimens from patients with CVDs (compared with individuals without CVDs) or diabetes (compared with individuals without diabetes)[260].

Finally, NOX5 is an important source of ROS in atherosclerosis. NOX5 is localized in the lesion area (in both endothelial and vascular smooth muscle cells) in the coronary arteries from patients with coronary artery disease undergoing cardiac transplantation[120]. Expression of NOX5 is very low in coronary arteries from patients undergoing cardiac transplantation who do not have coronary artery disease; however, NOX5 expression (both mRNA levels and protein levels) is significantly upregulated in patients with coronary artery disease undergoing cardiac transplantation[120]. Moreover, NOX5 has been shown to increase the proliferation of vascular smooth muscle cells, further suggesting a role of NOX5 in atherosclerosis[263]. So far, no direct evidence on the role of NOX5 in atherogenesis is available from animal models because rodents do not have NOX5.

Of note, eNOS uncoupling occurs in *Apoe*$^{-/-}$ mice[12,14,29]. Endothelial transgenesis of eNOS was reported to increase the formation of atherosclerotic lesions in *Apoe*$^{-/-}$ mice owing to increased eNOS uncoupling[18,264]. Strategies to recouple eNOS by supplementation with H_4B or endothelial-specific overexpression of GTPCH1 significantly reduced lesion formation in *Apoe*$^{-/-}$ mice, accompanied by decreased superoxide production, improved vasorelaxation and NO bioavailability, and reduced inflammation[14,18,19,25,264]. Angiotensin II induces atherogenesis in *Apoe*$^{-/-}$ mice, which was attenuated by the upregulation of eNOS phosphorylation and NO production when animals were fed with mitochondria-targeted aesculetin (6,7-dihydroxycoumarin)[265], implying a beneficial effect of eNOS recoupling on the prevention of atherogenesis in *Apoe*$^{-/-}$ mice. Angiotensin II infusion causes eNOS uncoupling in *Apoe*$^{-/-}$ mice, resulting in AAA formation[29]. In addition, NOX activation and NOX-derived ROS mediate angiotensin II-induced eNOS uncoupling in *hph-1* mice, leading to hypertension and AAA[27,32,34]. However, direct evidence as to whether eNOS uncoupling in atherosclerosis lies downstream of NOX activation requires further investigation.

Diabetic vascular complications

NOX activation has been implicated in endothelial dysfunction in diabetes. Expression of *NOX1* was induced by high glucose levels in human aortic endothelial cells in vitro in accordance with upregulation of superoxide production[257]. The superoxide production induced by high glucose levels was attenuated by *NOX1* siRNA or GKT137831 (REF.[257]). NOX activation has also been reported in animal models of both type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). In particular, we have shown that NOX1 protein levels were upregulated threefold in T1DM[26]. Knockdown of *Nox1* or *Noxo1* or deletion of *Ncf1* reduced eNOS uncoupling in STZ-induced diabetic animals[26]. However, knockout of *Nox2* or knockdown of *Nox4* did not alter eNOS uncoupling[26]. Taken together, these data indicate a NOX1-specific induction of eNOS uncoupling in T1DM. *Nox1* knockout also reversed the impaired endothelium-dependent vasodilatation in T1DM[26]. Blocking angiotensin II signalling in vivo with an AT_1 receptor antagonist or an ACE inhibitor attenuated NOX activity and eNOS uncoupling in STZ-treated T1DM mice[17]. In addition, *Nox1* deletion resulted in eNOS recoupling through preservation of DHFR function and restoration of H_4B bioavailability[26]. These findings indicate a pathological role of the angiotensin II–NOX1–eNOS uncoupling axis in the induction of vascular dysfunction in T1DM.

The roles of NOX activation and eNOS uncoupling in vascular dysfunction and inflammation have also been studied in *db*/*db* mice, a model of T2DM. These mice have impaired vascular relaxation compared with wild-type littermates, which is restored by treatment with apocynin[266]. Aortic mRNA and protein levels of $p22^{phox}$ and NOX1, but not NOX2, were elevated in *db*/*db* mice compared with wild-type mice, accompanied by increased superoxide production and impaired vasorelaxation[35,266], suggesting a role of NOX1 activation in inducing vascular dysfunction in T2DM. Additionally, eNOS uncoupling has been reported in *db*/*db* mice[35], indicating that NOX1-dependent eNOS uncoupling might be the cause of endothelial dysfunction in T2DM. However, the upstream mechanism of NOX1 activation in T2DM is different from that in T1DM. Circulating BMP4 levels were robustly elevated in both *db*/*db* mice and wild-type mice fed a high-fat diet (in contrast to the elevated angiotensin II levels in plasma observed in T1DM mice)[35]. Interestingly, noggin, a BMP4 antagonist, attenuates eNOS uncoupling and endothelial dysfunction in *db*/*db* mice[35], indicating BMP4-dependent eNOS uncoupling. siRNA targeting *Nox1* blocked BMP4-induced eNOS uncoupling[35]. These findings suggest that BMP4–NOX1 mediates eNOS uncoupling in T2DM. Furthermore, upregulation of the levels of the inflammatory regulators prostaglandin G/H synthase 2 (also known as COX2) and vascular cell adhesion protein 1 (VCAM1) in *db*/*db* mice was significantly blocked by noggin infusion[35]. Of note, BMP4-dependent COX2 upregulation was normalized by administration of sepiapterin, an eNOS-recoupling agent, indicating that COX2 lies downstream of BMP4-induced uncoupling of eNOS[35]. Taken together, these findings demonstrate that BMP4–NOX1-dependent eNOS uncoupling and subsequent COX2–VCAM1 activation mediate vascular dysfunction and inflammation in T2DM.

To examine whether vascular NOX-derived oxidative stress has a role in the development of obesity and metabolic syndrome, our group used transgenic mice with vascular smooth muscle-specific expression of *Cyba* that were fed a high-fat diet[242,267]. Of note, the *Cyba* transgene increased vascular superoxide and H_2O_2 production at baseline, which has a compensatory vasodilatory effect, such that the animals had no obvious pathological phenotype but a minimally increased blood pressure at baseline[242]. When fed a high-fat diet, these transgenic

mice developed marked obesity, insulin resistance, leptin resistance and glucose intolerance compared with wild-type mice[267]. The underlying mechanisms involve mitochondrial dysfunction and elevated mitochondrial ROS production in skeletal muscle, impaired spontaneous activity, as well as increased adipogenesis and perivascular inflammation[267]. Targeted deletion of *Cyba* in vascular smooth muscle prevented obesity and leptin resistance induced by a high-fat diet via restoration of skeletal muscle mitochondrial function and attenuation of adipogenesis and perivascular inflammation[267]. These findings are paradigm-shifting in establishing a novel concept that vascular-driven oxidative stress is a cause of obesity and metabolic syndrome rather than a consequence.

Diabetic vascular complications have been examined in both large vessels (such as the aorta) and small vessels (such as skeletal muscle arterioles and adipose microvessels). In human primary isolated skeletal muscle arterioles and human adipose microvascular endothelial cells, treatment with insulin induced vascular dysfunction via VAS2870-inhibitable superoxide production and eNOS uncoupling[268]. In this study, VAS2870 was used as a NOX2 inhibitor[268]. Although VAS2870 was later shown to be an inhibitor of all NOXs[269], these data suggest that insulin induces vascular dysfunction via NOX activation and superoxide production in small vessels. A thorough examination of three types of microvessels in *db*/*db* mice demonstrated that NOX activity (measured by lucigenin chemiluminescence in the presence of NADPH) was increased in diabetes in coronary arteries, mesenteric resistance arteries and femoral arteries[270]. This increased NOX activity was blocked by in vivo *Cyba*-targeted siRNA or SOD treatment[270]. In vivo knockdown of *Cyba* with siRNA also improved vascular relaxation in the three types of microvessels[270], again implying an intermediate role of NOX-dependent ROS production in mediating vascular dysfunction in diabetes.

Cardiac IR injury and MI

Cardiac IR induces ROS production when oxygen supply is restored after an ischaemic event[271–273]. Data have shown that NOX isoforms have important roles in IR injury. NOX2 protein levels were found to be elevated in cardiomyocytes from individuals who had died from acute MI[125]. In animal models, increased protein expression of NOX2 and NOX4 has been reported during IR (30 min of ischaemia followed by 24 h of reperfusion for in vivo experiments; 20–25 min of ischaemia followed by 1 h of reperfusion for ex vivo experiments)[31,58]. Reduced infarct size after IR was reported in mice with global knockout of *Nox2* or *Nox4* via inhibition of superoxide production[58]. Endothelial-specific deletion of *Smarca4*, which encodes the transcription factor BRG1 that regulates *Nox2* and *Nox4* transcription, attenuated IR-induced *Nox2* and *Nox4* expression, reduced superoxide production and decreased infarct size[274], indicating that endothelial NOX2 and NOX4 might have an important role in cardioprotection against IR injury. However, overexpression of NOX2 in cardiomyocytes or endothelial cells had no effect on infarct size in a mouse model of MI at 4 weeks[275]. Conversely, cardiac transgenesis of *Nox4* in mice increased oxidative stress and infarct size in response to IR using an ex vivo Langendorff system[276]. Consistently, protection from IR injury in mice with cardiac-specific *Nox4* knockout has been reported[58]. In accordance with these findings, we have shown that inhibition of NOX4 in vivo with the use of siRNA attenuated IR-induced infarct size in mice[31]. IR-induced upregulation of NOX4 levels increased cardiac ROS production, eNOS uncoupling and mitochondrial dysfunction, resulting in cardiac injury[31]. However, we did not observe cardioprotection in *Nox1*$^{-/-}$ or *Nox2*$^{-/-}$ mice subjected to IR[31], indicating a NOX4-specific role in IR injury. Our data established a critical role of the NOX4–uncoupled eNOS–mitochondrial dysfunction axis in mediating IR-induced cardiac injury[31]. Infusion of netrin-1 into Langendorff-perfused mouse hearts stimulates NO production from coupled eNOS, resulting in NO-dependent inhibition of NOX4, reduced oxidative stress, preserved mitochondrial function and markedly reduced infarct size[31].

Intriguingly, global *Nox2* knockout combined with cardiac-specific *Nox4* knockout resulted in increased cardiac injury in response to IR, despite reduced superoxide production[58]. A similar phenotype was observed in transgenic mice with cardiac-specific expression of a dominant-negative form of NOX4, which has been shown to suppress both NOX4 and NOX2 in cardiomyocytes[58,276]. The researchers suggested that mild downregulation of oxidative stress (by *Nox2* or *Nox4* deletion) is protective, whereas marked downregulation of oxidative stress (by combined *Nox2* and *Nox4* knockout or overexpression of a dominant-negative form of NOX4) increases cardiomyocyte death[58]. Markedly reducing the levels of oxidative stress is thought to lead to reduced levels of hypoxia-inducible factor 1α (HIF1α) and increased levels of peroxisome proliferator-activated receptor-α (PPARα) after IR[58,277]. Elevated levels of PPARα then stimulate free fatty acid metabolism, which in turn induces triglyceride accumulation in the heart and lipotoxicity[58]. Taken together, these studies suggest that a basal level of ROS can be cardioprotective and is required to maintain cardiac homeostasis, although high levels of ROS are deleterious and can result in MI[58,276,278].

Treatment with folic acid is reported to ameliorate ROS production and attenuate IR injury in rats[144], suggesting an important role of eNOS recoupling in cardiac protection against IR. Our studies have shown that NOX4, but not NOX1 or NOX2, was significantly upregulated and activated by IR, resulting in eNOS uncoupling, mitochondrial dysfunction and cardiac injury[31]. Given that NO effectively downregulates *NOX4* expression, loss of NO as a result of initial ROS production during IR leads to persistent NOX4 expression and activity[31]. We have previously reported that netrin-1 prevents IR-induced cardiac injury through elevated NO production ex vivo and in vivo[279–281]. This effect is via netrin receptor DCC-dependent activation of ERK1 (also known as MAPK3), ERK2 (also known as MAPK1) and eNOS[279,282]. Netrin-1–NO signalling also robustly inhibits the E3 ubiquitin-protein ligases SIAH1 and SIAH2 (which mediate the proteasome-dependent degradation of the netrin receptor DCC) thereby potentiating netrin-1-induced

cardioprotection[283]. In addition, netrin-1 inhibits post-MI autophagy to limit cardiac remodelling[31]. Therefore, netrin-1 abrogates IR-induced cardiac mitochondrial dysfunction and infarction through NO-dependent downregulation of *Nox4* expression and recoupling of eNOS[31]. These data indicate important crosstalk between NOX4, uncoupled eNOS and mitochondrial dysfunction in mediating cardiac IR injury, the interruption of which by netrin-1 is cardioprotective. Moreover, small netrin-1-derived peptides (9–11 amino acids) are highly effective in protecting against IR injury via production of NO, making these peptides a pharmacologically novel approach for the treatment of acute MI[284].

NOX5 is absent in rodents, but transgenic animals with the human isoform have been created. In a humanized mouse model with endothelial-specific (*Tie2* promoter-driven) knock-in of human *NOX5*, the IR-induced infarct size in the heart was not significantly different from that in wild-type controls, but the brain infarct size after stroke was increased through ROS-dependent blood–brain barrier leakage[247].

Heart failure

Heart failure is a chronic, progressive condition that often occurs as a result of maladaptive changes to compensate for cardiac hypertrophy[285]. The role of different NOX isoforms in the development of cardiac hypertrophy has been assessed in various genetically modified animal models, mostly focusing on NOX2 and NOX4 (REFS[286,287]). Multiple research groups have reported that NOX4 expression in the heart is upregulated in response to 2–4 weeks of transverse aortic constriction (TAC) to induce pressure overload, or after phenylephrine or angiotensin II infusion[89,134,240,288]. Indeed, cardiac-specific knockout of *Nox4* was found to be effective in attenuating TAC-induced or phenylephrine-induced cardiac hypertrophy[59,134], and cardiac-specific overexpression of *Nox4* potentiated the hypertrophic phenotype[59,134]. In animals with TAC-induced cardiac hypertrophy, NOX4 activation leads to mitochondrial superoxide production, resulting in apoptosis and cardiac dysfunction[59]. TAC-dependent and phenylephrine-dependent upregulation of NOX4 leads to superoxide accumulation in the nuclei and oxidation of histone deacetylase 4 (HDAC4)[134]. The oxidation of cysteine residues in HDAC4 induces cardiac hypertrophy through activation of nuclear factor of activated T cells (NFAT)[134,289]. Cardiac-specific overexpression of *Nox4* in mice potentiated angiotensin II-induced cardiac hypertrophy, which was significantly inhibited by GKT137831 administration[240]. The mechanisms of angiotensin II-induced hypertrophy involve upregulation of NOX4 levels, NOX4-dependent ROS production and subsequent increased phosphorylation of RACα serine/threonine-protein kinase (AKT)[240]. Phosphorylation of the two downstream effectors of AKT, mechanistic target of rapamycin (mTOR) and nuclear factor-κB (NF-κB; specifically, the p65 subunit), was upregulated in the hearts of angiotensin II-infused mice[240].

Angiotensin II has also been shown to promote H_2O_2 production in isolated cardiomyocytes[290]. In vivo generation of H_2O_2 in the heart induced heart failure in rats[291]. In this study, cardiac H_2O_2 was produced from the conversion of orally administered D-alanine to pyruvate, catalysed by a virally delivered D-amino acid oxidase (driven by the *Tnnt2* promoter)[291]. Interestingly, studies have shown that ageing or TAC impaired cardiac function in mice with cardiac-specific overexpression of *Nox4*, without significant changes in cardiac hypertrophy at the organ level[48,78]. However, left ventricular cardiomyocyte cross-sectional size was increased in *Nox4*-transgenic mice[59,89], suggesting a compensatory response of the heart against cardiac dysfunction. The inconsistent phenotypes of *Nox4* transgenesis in cardiac hypertrophy models might be caused by different time points of *Nox4* expression induction in these animal models[59,89,240]. In the inducible transgenic model (in cardiomyocytes), *Nox4* expression was induced 7 days before angiotensin II infusion[240]. In this model of transient overexpression of *Nox4* in the heart, NOX4 exacerbated angiotensin II-induced hypertrophy via increased ROS production[240]. By contrast, in animals with embryonic cardiac overexpression of *Nox4*, no further exaggeration in hypertrophy was developed after TAC, suggesting that adaptation to *Nox4* overexpression had been established in these animals[59,89,134].

Of note, another research group observed that global knockout of *Nox4* exaggerated hypertrophy during exposure to chronic pressure overload (6 weeks of suprarenal aortic constriction)[288]. Deletion of *Nox4* inhibited expression of *Hif1a* and *Vegf*, which blocked angiogenesis of myocardial capillaries and contributed to hypertrophy[288]. In addition to the different strategies applied to induce hypertrophy, the discrepancy between this global knockout model[288] and the previously discussed cardiac-specific knockout model[59,134] suggests that NOX4 in different cell types might have opposing roles in the development of hypertrophy, which warrant further investigation.

The role of NOX2 in the development of cardiac hypertrophy has also been studied. At a lower dose of angiotensin II infusion (0.3 mg/kg per day), which did not alter blood pressure, *Nox2* deletion protected against angiotensin II-induced cardiac hypertrophy through reduced ROS production[292]. The protein level of NOX2 was reportedly not modulated by angiotensin II in the mouse heart[240]. These data suggest that angiotensin II induces hypertrophy through activation of NOX2 rather than upregulation of its expression level. Moreover, cardiac NOX2 protein levels were reported to be upregulated after aortic banding[293,294] or MI[295,296]. Of note, NOX2 protein levels were elevated in left ventricular myocardial tissue from patients with end-stage heart failure and dilated cardiomyopathy compared with that of individuals without heart failure[294]. The same research group has reported that deletion of *Nox2* in mice prevented TAC-induced oxidative stress and development of hypertrophy through inhibition of MAPK signalling[294], suggesting a pathogenic role of NOX2 in heart failure. Similarly, a potential role of NOX2 in MI-induced hypertrophy was also studied in genetically modified *Nox2* mice. Global knockout of *Nox2* protected against post-MI cardiac hypertrophy and restored cardiac function[295]. As expected, cardiac-specific overexpression of *Nox2* increased chronic MI (left coronary artery

ligation)-induced cardiomyocyte hypertrophy compared with wild-type littermates, in accordance with elevated superoxide production, whereas endothelial-targeted overexpression of *Nox2* did not alter cardiac remodelling after MI[275], indicating that cardiac but not endothelial NOX2 is involved in cardiac remodelling after MI.

eNOS uncoupling has been reported to occur in mouse hearts in response to TAC[16,297]. Supplementation with H_4B recoupled eNOS in wild-type mice after TAC, whereas knockout of *Nos3* (encoding eNOS) reduced TAC-induced hypertrophy and cardiac remodelling[16,145]. These results are consistent with the observation that NOX2-dependent and NOX4-dependent ROS production has an important role in the development of cardiac hypertrophy and that NOX-derived ROS lead to eNOS uncoupling. Another downstream effector of NOX-derived ROS is mitochondria. Excessive ROS production derived from mitochondria has been shown to contribute to heart failure[124,179,297–299]. Treatment of a healthy myocardium sample from dogs with antimycin A (a mitochondrial complex III inhibitor) reproduced the increase in superoxide production seen in the failing heart[298]. In angiotensin II-infused animals, ROS scavenging with *N*-acetylcysteine was less effective than mitochondria-targeted scavenging with peptide SS-31 in the prevention of cardiac hypertrophy, suggesting that mitochondrial ROS have an important role in modulating cardiac remodelling in angiotensin II-infused animals[183,300]. A positive correlation has been reported between myocardial ROS and left ventricular contractile dysfunction[298]. In a guinea pig model, heart failure was induced by ascending aorta constriction and daily infusion of the β-adrenergic agonist isoprenaline[299,301]. With an adenovirus-delivered H_2O_2 sensor (roGFP-ORP1) targeting the cytoplasm and mitochondria, the researchers showed that levels of both cytoplasmic and mitochondrial ROS were significantly increased in freshly isolated cardiomyocytes from failing hearts compared with cardiomyocytes from sham control hearts[299,302]. Administration of MitoTEMPO to clear mitochondrial ROS effectively normalized both cytoplasmic and mitochondrial ROS in cardiomyocytes from failing hearts[299], indicating crosstalk between mitochondria and oxidases in the cytoplasm. Administration of MitoTEMPO from the day of ascending aorta constriction or after heart failure is already present (3 weeks after the surgery) prevented or reversed heart failure[299], suggesting an important role of mitochondrial ROS in the development of heart failure. Taken together, these results indicate that eNOS uncoupling and mitochondrial ROS production contribute to NOX activation-induced pathogenesis of heart failure.

Cardiac arrhythmias

Cardiac arrhythmia is associated with elevated production of ROS. A growing number of studies have shown that NOXs are emerging sources of excessive production of ROS in the pathogenesis of arrhythmia[53,103,303,304]. A study in pigs showed increased NOX2 expression after MI in line with the development of arrhythmia, whereas reducing NOX2 protein levels by acute unloading of the left ventricle decreased the incidence of arrhythmia[296]. Atrial fibrillation (AF) is the most common cardiac arrhythmia. Expression of NOX2 and NOX4 and production of ROS were found to be upregulated in atrial tissue from patients with AF compared with that from individuals in sinus rhythm[303,304]. We have previously reported upregulated NOX4 expression and H_2O_2 levels (the detectable product of NOX4) in the cardiac tissues of patients with AF, whereas NOX2 expression was not significantly changed[304]. However, a potential causal role of NOX4 activation in the development of AF remained uncertain. To address this question, we examined arrhythmogenesis in zebrafish embryos with acute induction of *nox4* (REF.[60]). Overexpression of *nox4*, instead of *nox2*, induced ROS production and cardiac arrhythmia (in the form of irregular heartbeats) in zebrafish embryos, both of which were blocked by *nox4* antisense morpholino oligonucleotide co-injection, treatment with poly(ethylene glycol)-SOD or NOX4 inhibitors 6-(dimethylamino)fulvene, fulvene-5 or proton sponge blue[60]. Overexpression of Nox4-P437H protein, a dominant negative form of Nox4, did not induce ROS production or an arrhythmic phenotype[60]. *Nox4* overexpression induced arrhythmia through ROS-dependent activation of CaMKII[60]. These data demonstrate a causal role of Nox4-derived ROS in arrhythmogenesis in zebrafish[60]. The emerging roles of NOXs as a source of ROS production in inducing the development of AF has been previously reviewed[53].

Moreover, uncoupled eNOS and mitochondrial dysfunction have been reported to be involved in cardiac arrhythmia. Pretreatment with a NOS inhibitor significantly (although to a lower extent than a NOX inhibitor) reduced superoxide production in atrial homogenates from patients with AF, implicating uncoupling of eNOS[303]. Additionally, a mitochondrial complex I inhibitor lowered the basal levels of superoxide production in atrial homogenates from patients with AF, suggesting that mitochondrial ROS might contribute to the development of AF[303]. These data suggest that uncoupled eNOS and mitochondrial dysfunction are likely to underlie the development of AF downstream of NOX activation.

NOX inhibitors

Given the critical roles of NOXs in the pathogenesis of CVDs, they have been considered as prominent targets for the development of novel therapeutic agents[305]. The long quest to develop specific NOX inhibitors is illustrated in BOX 2.

Small-molecule inhibitors

Initially, several nonspecific small molecules were used as NOX inhibitors[306]; diphenyleneiodonium (DPI) and apocynin are the most widely studied[28,213,307–310]. DPI is a general, nonreversible inhibitor of all NOX isoforms with an inhibitory constant (K_i) of 10–70 nmol/l (REFS[311–313]). However, off-target effects have been reported with DPI, which also inhibits other ROS-generating enzymes and systems, including XO, NOS and the mitochondrial electron transport chain[314–318]. Apocynin inhibits the membrane translocation of $p47^{phox}$ and $p67^{phox}$ (REFS[102,319–321]) in leukocytes and was initially considered to be a specific

inhibitor of NOXs (but was later found to inhibit all flavin-containing enzymes). Apocynin is activated after the formation of apocynin dimers in leukocytes[321,322]; however, in endothelial and smooth muscle cells, these dimers do not form and, therefore, apocynin is not activated in these cell types[322]. Nevertheless, apocynin has intrinsic antioxidant activity as a ROS scavenger, which explains the inhibitory effects of apocynin treatment on ROS levels in endothelial cells and smooth muscle cells[102,318,322,323].

Other small-molecule NOX inhibitors have also been investigated and used. 4-(2-Aminoethyl)-benzenesulfonyl fluoride, an inhibitor of serine proteases, has been used as a NOX inhibitor[324,325]. As a nonspecific inhibitor, 4-(2-aminoethyl)-benzenesulfonyl fluoride has low inhibitory potency for NOX (100 μmol/l) compared with DPI (10 μmol/l) and is, therefore, not frequently used[102,326]. A synthetic polyphenol, S17834, reduced tumour necrosis factor-induced NOX activity in endothelial cells and inhibited atherosclerotic lesion formation in the aortas of *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice[327,328]. S17834, which targets 5′ AMP-activated protein kinase[102], did not change superoxide production by XO and is not a scavenger of superoxide. Statins — inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase that are used for the lowering of blood lipid levels — inhibit NOX activity by targeting RAC1 (REFS[329–332]). However, statins also upregulate eNOS and NO production[333]. NSC23766, a RAC1 inhibitor, has been used as a NOX inhibitor[15,334,335]. VAS2870 and VAS3947 have also been used as NOX inhibitors[336–338]. Whereas VAS2870 inhibits NOX2, NOX4 and NOX5, VAS3947 targets NOX1, NOX2 and NOX4 (REF.[102]). The lack of specificity of these two inhibitors has limited their use for isoform-specific evaluation of NOXs in disease development. An inhibitor thought to be selective for NOX1, 2-acetylphenothiazine (known as ML171), was identified by high-throughput screening[316,323]; however, this inhibitor was soon demonstrated to inactivate all NOX isoforms, including NOX1, and interferes with the Amplex Red Assay for H_2O_2 detection[339]. Owing to their chemical similarity to DPI, triphenylmethane dyes have been tested for their NOX inhibitory capacity[340]. Brilliant green and gentian violet have displayed effective inhibition of NOX2 and NOX4, and imipramine blue inhibited NOX4-derived ROS in vitro[341]. In addition, fulvene derivatives have been synthesized and been shown to inhibit NOX2 and NOX4 activity in vivo[342]. In particular, 6-(dimethylamino) fulvene, fulvene-5 and proton sponge blue blocked NOX4-induced ROS production in vivo[60].

Peptide-based inhibitors

Peptide-based inhibitors have been designed to target NOXs. NOX2 docking sequence (ds)-tat (originally known as gp91ds-tat) is a chimeric peptide designed to interfere with the interaction between NOX2 and p47^{phox} (REF.[343]). NOX2ds-tat contains nine amino acids (NOX2ds) corresponding to the human and mouse NOX2 sequence but with one substituted amino acid (isoleucine for valine at position 89)[343]. To ensure the in vivo delivery of this peptide, a specific nine-amino acid peptide of the human immunodeficiency virus coat (HIV-tat) was linked[343]. NOX2ds-tat completely inhibited angiotensin II-induced aortic superoxide production and blood pressure increase, suggesting effective attenuation of angiotensin II-induced NOX activation[343]. The specificity of peptide NOX2ds has been examined by the same researchers, who showed that NOX2ds did not change the activity of NOX1, NOX4 or XO; NOX2ds is, therefore, considered to be a specific inhibitor of NOX2 (REF.[344]). Similarly, an 11-amino acid peptide, NOXA1ds, which mimics the activation domain of NOXA1, was designed to block NOX1 activity (without fusion to another peptide to facilitate cellular delivery)[345]. In this peptide, the phenylalanine at position 199 was changed to alanine[345]. NOXA1ds disrupted the association between NOX1 and NOXA1 and specifically inhibited NOX1-dependent superoxide production, without changing ROS production by NOX2, NOX4, NOX5 or XO[345]. NOXA1ds significantly inhibited hypoxia-induced or angiotensin II-induced ROS production in endothelial cells and vascular smooth muscle cells, respectively[132,345]. NOXA1ds also blocked vascular endothelial growth factor (VEGF)-induced wound healing in cultured endothelial cells[345]. Given that both NOX2ds-tat and NOXA1ds are peptide-based inhibitors that are subject to degradation in the gut, further modifications might be needed to improve their oral bioavailability and efficacy[345,346]. Given the limitations of the existing agents, the development of new classes of NOX inhibitor is urgently needed. Ideally, an agent should inhibit a specific NOX isoform, because different NOX isoforms are selectively involved in the pathogenesis of different CVDs.

Small-molecule inhibitors of GKT family

Several novel small molecules have been identified and characterized as NOX inhibitors[316,347–350]. These inhibitors seem to target NOX isoforms selectively. During a high-throughput screening campaign, several potent pyrazolopyridine dione derivatives were identified as NOX4 inhibitors[347]. Following investigation of the structure–activity relationship around the pyrazolopyridine dione core, two NOX inhibitors (GKT136901 and GKT137831) were discovered[347]. These inhibitors preferentially target NOX1, NOX4 and NOX5 (K_i = 10–100 nmol/l) over other NOX isoforms (K_i > 1 μmol/l for NOX2 and K_i > 100 μmol/l for XO)[311–313,351,352]. In one report, vascular smooth muscle cell-specific overexpression of human *NOX5* increased vascular contractile function, which was blocked by administration of *N*-acetylcysteine, a ROS scavenger, but not by GKT137831 (10 μmol/l)[245]. These data suggest that GKT137831 preferentially targets NOX1 and NOX4 over NOX5 (REF.[245]). GKT136901 and GKT137831 did not show off-target effects when tested on G protein-coupled receptors, kinases, ion channels and other ROS-producing and redox-sensitive enzymes[312,313], making them very specific inhibitors of NOX1 and NOX4. Further studies have shown that GKT136901 did not interact with NO, superoxide or hydroxyl radicals[353], but scavenged peroxynitrite through direct interaction[353] and decreased the Amplex Red fluorescent signal[102], weakening its potential as a selective and direct NOX inhibitor, although the clearance of free radicals might

be beneficial. GKT136901 was also reported to inhibit DUOX-dependent ROS production at micromolar concentrations in cells[354]. The capacity of GKT137831 to clear free radicals directly has not been reported.

Both GKT136901 and GKT137831 showed high plasma concentrations in vivo[347,355]. The half-life of GKT137831 is longer than that of GKT136901 in both rodents and humans[355,356]. Oral administration of GKT136901 (10 mg/kg of body mass) in *Apoe*$^{-/-}$ mice fed a high-fat diet reduced aortic lesion formation to a similar level to that previously reported in *Apoe*$^{-/-}$*Nox1*$^{-/-}$ double knockout animals[256,357]. This decrease was accompanied by reduced aortic superoxide production and systematic oxidative stress in GKT136901-treated *Apoe*$^{-/-}$ mice[357]. Likewise, administration of GKT137831 (60 mg/kg of body mass) in STZ-induced diabetic *Apoe*$^{-/-}$ mice completely reduced aortic lesion size to basal levels[257,358]. Similarly, treatment with GKT137831 mimicked the anti-atherosclerotic effect of *Nox1* deletion in reducing aortic superoxide production, aortic inflammation and fibrosis[257,358]. In the same study, *Nox4* deletion was also examined but had no effect on atherosclerosis[257], indicating the potency of GKT137831 for NOX1 inhibition and a selective role of NOX1 in atherogenesis in this model. This observation is consistent with our findings that NOX1 has an essential role in inducing vascular dysfunction in mice with either T1DM or T2DM[26,35].

Moreover, the effect of GKT137831 has been compared with that of *Nox4* deletion on cardiac remodelling in vivo. Angiotensin II is known to induce cardiac remodelling in wild-type mice[51,52,240]. In mice with cardiac-specific overexpression of *Nox4*, infusion of angiotensin II induced severe cardiac remodelling and fibrosis through ROS production and the AKT–mTOR and NF-κB signalling pathways[240]. Administration of GKT137831 (40 mg/kg per day) abolished the increase in oxidative stress, suppressed AKT–mTOR and NF-κB signalling, and attenuated cardiac remodelling and fibrosis[240]. These data suggest that GKT137831 is a potent inhibitor of NOX4 in vivo.

The effects of GKT compounds have also been examined in angiogenesis. In mouse cultured primary lung endothelial cells, either administration of GKT136901 or *Nox1* deletion inhibited VEGF-induced ROS production[311]. In vivo data in mice showed that GKT136901 administration (40 mg/kg per day) was more potent than *Nox1* deletion in inhibiting angiogenesis in implanted Lewis lung carcinoma 1 tumours[311]. In addition, GKT137831 is currently under phase II clinical testing in patients with T2DM and albuminuria (NCT02010242) and patients with primary biliary cholangitis receiving ursodeoxycholic acid (NCT03226067). Therefore, GKT137831 is one of the most promising NOX inhibitors to date.

Newly reported inhibitors

GLX compounds were identified as NOX inhibitors by high-throughput screening[348,349]. GLX351322 was reported to inhibit NOX1, NOX2, NOX4 and NOX5 (REFS[348,349]). However, its lack of specificity greatly reduced its potential application. Two novel GLX compounds (GLX481372 and GLX7013114) have been identified as NOX4 inhibitors and tested in vitro[349]. These GLX compounds were also identified by high-throughput screening and were developed in a structure–activity relationship campaign[349]. Whereas GLX481372 selectively targets NOX4 and NOX5, GLX7013114 is, so far, the first-reported highly selective inhibitor of NOX4 (REF.[349]); GLX7013114 at concentrations <1 μmol/l reportedly inhibits only NOX4 (REF.[349]). GLX7013114 protects islet cells against high glucose+palmitate and cytokine-induced cell death[349]. The researchers believe that GLX7013114 targets a unique domain of NOX4 that makes it selective in inhibiting NOX4, whereas previous NOX inhibitors target a common site shared by more than one NOX isoform[349]. GLX7013114 did not show activity as a direct ROS scavenger or as an inhibitor of XO or glucose oxidase[349].

GSK2795039 is the first small molecule identified that selectively inhibits NOX2 over other NOX isoforms[350]. GSK2795039 does not show inhibition of PKC, XO or eNOS at concentrations that are efficacious for NOX2 inhibition[350]. Systemic administration of GSK2795039 (intraperitoneal injection of 100 mg/kg) mimics the effect of *Nox2* deletion in the attenuation of ROS production in mice in vivo[350]. Further investigation is needed to characterize GSK2795039 as a selective inhibitor of NOX2. A nonspecific NOX inhibitor, APX-115, has been shown to inhibit NOX1, NOX2 and NOX4 in animal models in vivo and to protect against kidney injury in animal models of T1DM and T2DM[359–361].

Epigenetic modulation of NOXs

Methylation-dependent and acetylation-dependent regulation of NOX gene expression has been studied. Methylation-mediated downregulation of *DUOX1*, *DUOX2* and *NOX5* expression has been reported in cancer cells[362–364]. Incubation with a methyltransferase inhibitor (5′-aza-2′-deoxycytidine) increased *DUOX1*, *DUOX2* and *NOX5* expression in cancer cells[362,363].

Conversely, deficiency of the histone acetyltransferase KAT2A dramatically downregulated *NOX2* transcription and superoxide production[365]. In addition, inhibition of HDACs with pharmacological inhibitors (scriptaid, suberoylanilide hydroxamic acid, trichostatin A and valproic acid) reduced *NOX1*, *NOX2*, *NOX4* and *NOX5* mRNA levels and ROS production[366–369]. The underlying mechanism involves HDAC inhibitors decreasing binding of the histone acetyltransferase p300 to the NOX promoter regions, reducing accessibility of RNA polymerase II and attenuating transcription efficiency[368]. HDAC inhibitors also increased expression of *SOD3* through acetylation and methylation of histones in its promoter region[367]. Therefore, epigenetic modulation regulates oxidative stress via both NOXs and SOD.

Surprisingly, application of suberoylanilide hydroxamic acid (an HDAC inhibitor) inhibited STZ-induced upregulation of *Nox1*, *Nox2* and *Nox4* expression as well as ROS production in mouse aorta[369]. This change is caused by inhibition of glucose-stimulated interaction of HDAC1, HDAC2 and p300 with the promoter regions of *NOX1*, *NOX4* and *NOX5*, and of glucose-stimulated acetylation and *NOX1*, *NOX4* and *NOX5* transcription

in human vascular smooth muscle cells[369]. In endothelial cells, high glucose levels activate hydroxymethylating enzymes, leading to increased levels of 5-hydroxymethylcytosine (generated from 5-methylcytosine) and its binding to the *RAC1* promoter region as well as activation of *RAC1* transcription in diabetes[370]. Moreover, in an animal model of cardiac IR injury, macrophage expression of myocardin-related transcription factor A recruited the histone acetyltransferase KAT8 to the promoters of *Nox1*, *Nox2* and *Nox4* to activate transcription[371]. Inhibition of KAT8 with MG149 significantly downregulated *Nox1* and *Nox4* expression and ROS production and restored myocardial function in mice exposed to IR injury[371]. These studies have revealed an emerging mechanism of epigenetic regulation of NOX gene transcription and NOX-derived ROS production in CVDs[369–371], the targeting of which might provide alternative therapeutic strategies to NOX inhibition.

In addition to the challenge of developing isoform-specific inhibitors of NOXs, it is important to note that basal ROS production is required for many physiological processes, as discussed above[2,58]. Removing basal levels of ROS might compromise these physiological functions. Furthermore, tissue-specific or cell-specific roles of NOX isoforms need to be targeted in different cardiovascular conditions. Therefore, details of NOX inhibitor application (that is, timing, dosing and cell-specific or tissue-specific targeting) are of great importance in the prevention and treatment of CVDs.

Conclusions

During the pathogenesis of CVDs, ROS are generated by various sources, particularly NOXs. The interactions between different ROS-producing systems demonstrate a critical role of NOXs and NOX-dependent activation of secondary oxidase systems in sustaining oxidative stress, leading to the development of CVDs. Upon activation, NOX-derived ROS induce eNOS uncoupling, mitochondrial dysfunction and, to a lesser extent, XO activation, resulting in further release of ROS and tissue injury. Systematic evaluations of interactions between ROS-producing systems have provided new insights into the mechanistic details of CVDs, especially isoform-specific activation of NOXs under different disease conditions. Targeting specific NOX isoforms selectively to correct eNOS uncoupling and mitochondrial dysfunction might prove to be highly beneficial as a novel therapeutic strategy for the treatment of various CVDs, including hypertension, aortic aneurysms, diabetic vascular dysfunction, atherosclerosis, cardiac IR injury, heart failure and cardiac arrhythmias. Therefore, the development of novel, effective and isoform-specific NOX inhibitors, as well as the development of novel strategies targeting uncoupled eNOS and mitochondrial dysfunction, are essential in realizing the therapeutic value of targeting NOX isoforms and downstream oxidase systems for the prevention and treatment of CVDs.

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Competing interests
The authors declare no competing interests.

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