

Differential roles of NADPH oxidases in vascular physiology and pathophysiology

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. NADPH oxidases
4. Regulation of vascular tone
5. Vascular remodeling and extracellular matrix reorganization
6. Phenotypic modulation (proliferation and differentiation)
7. Migration
8. Apoptosis and senescence
9. Hypoxia
10. Pulmonary hypertension
11. Atherosclerosis
12. Angiogenesis
13. Diabetic vascular disease
14. Perspective
15. Acknowledgments
16. References

1. ABSTRACT

Reactive oxygen species (ROS) are produced by all vascular cells and regulate the major physiological functions of the vasculature. Production and removal of ROS are tightly controlled and occur in discrete subcellular locations, allowing for specific, compartmentalized signaling. Among the many sources of ROS in the vessel wall, NADPH oxidases are implicated in physiological functions such as control of vasomotor tone, regulation of extracellular matrix and phenotypic modulation of vascular smooth muscle cells. They are involved in the response to injury, whether as an oxygen sensor during hypoxia, as a regulator of protein processing, as an angiogenic stimulus, or as a mechanism of wound healing. These enzymes have also been linked to processes leading to disease development, including migration, proliferation, hypertrophy, apoptosis and autophagy. As a result, NADPH oxidases participate in atherogenesis, systemic and pulmonary hypertension and diabetic vascular disease. The role of ROS in each of these processes and diseases is complex, and a more full understanding of the sources, targets, cell-specific responses and counterbalancing mechanisms is critical for the rational development of future therapeutics.

2. INTRODUCTION

Virtually all resident cells in the vessel wall produce and respond to reactive oxygen species (ROS) (1-4). Vascular smooth muscle cells (VSMCs) in response to stimuli change phenotype from quiescent/contractile to synthetic, being able to proliferate, migrate and synthesize extracellular matrix. Endothelial cells respond to vasoactive agents by releasing nitric oxide (NO) to regulate vascular tone, by maintaining the pro/antithrombotic balance, and by promoting or inhibiting permeability and inflammation. Adventitial fibroblasts can influence vessel contraction and medial hypertrophy. ROS have been recognized as key regulators of many of these responses. More recently, it has been recognized that redox processes occur in a compartmentalized manner, with generation of different ROS in distinct organelles by several cellular enzyme systems (5-7). Vascular cells have diverse sources of ROS, namely from oxidative ATP generation in the mitochondria, xanthine oxidase during the metabolic pathway for acid uric formation, oxidoreductases such as cytochrome P450, glutathione peroxidase and myeloperoxidase, multiple thiol-disulfide exchanges during folding of nascent proteins in the endoplasmic reticulum, uncoupled nitric oxide synthases and Nox family NADPH

Nox enzymes in vascular function

Table 1. Nox enzymes in the vasculature

Nox member	localization	ROS type	Regulators	Expression	Activation	Cardiovascular physiology	Cardiovascular pathophysiology
Nox1							
	Transmembrane	Superoxide	P22phox	Smooth muscle cells	Stimulus induced	Proliferation, migration	Vascular hypertrophy
	Caveolae	Anion	P47phox, nox1, rac	Epithelial cells, uterus	Ang ii, pdgf, fgf	Extracellular matrix production	Hypertension, vascular injury
	Endosomes		Or nox1, nox1, rac	Osteoclasts, neurons		Host defense	Diabetes
Nox2							
	Phagosomes	Superoxide	P22phox, p47phox,	Endothelial cells	Agonist-induced	Angiogenesis	Hypoxia, diabetes
	Caveolae	Anion	P67phox, p40phox	Cardiomyocytes, hepatocytes	(ang ii, vegf, tnfa, endothelin1)	Apoptosis, oxygen sensing	Hypercholesterolemia
	Endosomes		Rac	Fibroblasts, phagocytes	Mechanical forces	Inflammation	Pulmonary hypertension
				Neurons, skeletal muscle myocytes			
Nox3							
	Plasma membrane	Superoxide	P22phox, nox1	Inner ear	Agonist-induced (tnfa)		
		Anion	Possibly Nox1, Rac	Lung endothelial cells	Constitutively active	Unknown	Insulin resistance
				Hepatocytes			
Nox4							
	Focal adhesion	Hydrogen	P22phox	Mesangial cells, smooth muscle	Constitutively active	Senescence, apoptosis	Hypertrophy
	Nucleus	Peroxide	Poldip2	Endothelial cells, fibroblasts	Stimulus induced	Endoplasmic reticulum stress	Pulmonary hypertension/fibrosis
	Endoplasmic reticulum			Keratinocytes, osteoclasts	(tgfb, il-1, vit.d, thrombin,	Survival, differentiation	Metabolic syndrome
				Neurons, hepatocytes	ER stressors)	Oxygen sensing	
				Cardiomyocytes		Cytoskeletal regulation	
Nox5							
	Internal membranes	Superoxide	Calcium	Endothelial cells	Calcium activated	Proliferation	Unknown
	Plasma membrane	Anion		Smooth muscle cells		Inflammation	

oxidases (3, 5, 8-12). Maintaining the proper local balance of ROS production and removal is essential for normal physiology, with excess ROS potentially contributing to disease development (13). This review will focus on the role of ROS in both of these processes, with particular emphasis on NADPH oxidase-derived signaling.

3. NADPH OXIDASES

The Nox protein family is a major source of ROS in vascular cells (8, 14). The first member of this family to be described was gp91phox or Nox2, which generates large amounts of ROS in phagocytic cells, acting as an important component of innate immunity (15). In 1999, mox1 or NOH-1 (now called Nox1), a homolog of gp91phox, was discovered and was identified as an enzymatic source of ROS in non-phagocyte cells (16, 17). Since then seven members of the Nox family have been identified (Nox1-Nox5, Duox1 and Duox2) (2, 18). These isoforms differ from one another in their patterns of expression, subcellular localization, type of ROS produced and mode of activation (Table 1).

The Nox proteins generate ROS by transfer of a single electron from NADPH to molecular oxygen (18). The proteins have an oxidoreductase structure with a transmembrane region that contains two heme groups and a cytosolic portion with NADPH and FAD binding sites (8, 18-20). Nox1-4 require p22phox for their activity while Nox5 does not (20-22). p22phox is a regulatory membrane protein that functions as a docking site for regulatory proteins (p40phox, p47phox and p67phox for Nox2; Nox1 and Nox1 for Nox1 and 3; polymerase delta interacting protein 2 (Poldip2) for Nox4) (2, 10, 23-27). The small

GTPase Rac also regulates Nox1-3 activity (8, 23, 28-30). Nox5 also differs from the other Nox enzymes in that it does not require any cytosolic subunits (31). Structure predicts that all isoforms release superoxide (O_2^-); however, while this is true for Nox1, Nox2, Nox3 and Nox5, Nox4 and the Duox enzymes release mostly hydrogen peroxide (H_2O_2) (8, 32). In vascular cells, Nox1, Nox2, Nox4 and Nox5 have been linked to several pathophysiological conditions such as hypertension, atherosclerosis, restenosis after injury and diabetic vasculopathy (32-36).

Nox1, found in plasma membrane, caveolae and endosomes in VSMCs, is induced by growth factors and vasoactive agents and activated by Src-dependent, protein kinase C (PKC)-mediated p47phox phosphorylation (18, 37, 38). Nox2 is present in plasma membrane and is activated similarly to vascular Nox1 (14, 39). Nox4 is thought to be constitutively activated and its activity is correlated with its level of expression (40). However, recent studies show that Nox4 is regulated by Poldip2 and such regulation increases its activity, although the mechanism remains to be determined (24). This novel partner colocalizes with p22phox and Nox4 at focal adhesions and in the nucleus of VSMCs (24). Nox4 is also found in the perinuclear space, endoplasmic reticulum and plasma membrane (37). Another potential regulatory mechanism for Nox4 (and Nox1) involves a chaperone of the endoplasmic reticulum, protein disulfide isomerase (PDI) (41). Downregulation of PDI decreases the Nox activity induced by angiotensin II (Ang II), which has previously been attributed to Nox1 (42). For Nox4, however, the relationship with PDI may be due to an interaction between oxidative and endoplasmic reticulum

stress (43-45). Nox5 is regulated by elevation of calcium levels, and is found in the cytoskeletal fraction, endoplasmic reticulum and plasma membrane. Like Nox5, Duox enzymes are activated directly by calcium, although they do not appear to be expressed in the vasculature (8, 10, 14, 18, 20, 22, 46).

4. REGULATION OF VASCULAR TONE

Nox-derived ROS have physiological and pathophysiological roles in the cardiovascular system. As noted, Nox enzymes promote cell growth, migration, proliferation and differentiation, and these effects have been related with pathophysiological conditions. However, Nox enzymes are not important only in cell injury conditions, but also during development, reparative processes and in the physiology of the vascular tone and oxygen sensing (8, 47).

The endothelial regulation of vascular tone is directly correlated with local production and activity of NO. This regulation is sensitive to any alterations in local stimuli such as shear stress or the presence of local vasodilators. In response to these stimuli, increases and redistribution of blood flow occur in specific vascular networks (48-50). The presence of $O_2^{\cdot-}$ in this environment is an important foil to NO action, because $O_2^{\cdot-}$ can rapidly interact with NO to form peroxynitrite (51, 52). Consequently, the bioavailability of NO is reduced and other reactive species are generated. Peroxynitrite can 1) uncouple endothelial nitric oxide synthase (eNOS) by oxidizing tetrahydrobiopterin (BH_4) thus leading to reduced NO and increased $O_2^{\cdot-}$; 2) inactivate prostacyclin synthase (an important source of endothelium-derived relaxing factor); and 3) inhibit superoxide dismutases, inducing oxidative stress (53, 54).

Conversely, it has been shown that H_2O_2 can induce and activate eNOS (55, 56). H_2O_2 can also exert vasodilatory effects in vascular cells by hyperpolarization mechanisms (55-59). Recently, endothelial H_2O_2 has been proposed to be the endothelium-dependent hyperpolarization factor (EDHF) stimulated by shear stress to induce relaxation of the vessel (60, 61). Moreover, vascular overexpression of catalase counteracts the activity of some vasoconstrictor agents, suggesting that endogenous H_2O_2 may be an important regulator of blood pressure (62). On the other hand, other studies report that catalase has minimal effects on endothelium dependent relaxation in aorta and small arteries (63). Adventitia-derived H_2O_2 has been proposed to impair vascular relaxation via p38 mitogen-activated protein kinase (MAPK) activation and SH₂ domain-containing protein tyrosine phosphatase-2 (SHP2) inhibition. Other findings implicate H_2O_2 -dependent extracellular signal regulated kinase (ERK) 1/2 activation in inhibiting relaxation (58, 64, 65).

It is thus clear that ROS critically regulate vascular tone, but that their effects may be dependent upon the identity of ROS, the nature of the stimulus and the vascular bed upon which it acts. For example, H_2O_2 relaxes coronary and pulmonary arteries but may relax or

constrict mesenteric arteries, while $O_2^{\cdot-}$ inactivates NO to counteract relaxation and may directly constrict vessels (66). In human coronary arterioles, bradykinin-induced dilation is reduced by the Nox2 inhibitor gp91ds-tat, suggesting that Nox2 mediates this response, but the relevant ROS is H_2O_2 , which reduces the bioavailability of epoxyeicosatrienoic acids (67). Moreover, in this arterial bed, flow induces endothelial production of H_2O_2 , which activates large conductance Ca^{2+} -activated K^+ channels on the smooth muscle cells to induce relaxation (59). The multiple roles of ROS complicate the potential usefulness of Nox inhibitors in blood pressure regulation, since reduction of $O_2^{\cdot-}$ would favor improved relaxation and thus a lowering of blood pressure, but reduction of H_2O_2 might have the unwanted side effect of impairing coronary vasodilation. Because Nox1/2 generate $O_2^{\cdot-}$ while Nox4 releases mostly H_2O_2 , identifying which Nox is responsible for which response may allow us to specifically target the offending Nox in specific diseases.

5. VASCULAR REMODELING AND EXTRACELLULAR MATRIX REORGANIZATION

Vascular remodeling refers to a structural modification that results in changes in wall thickness and lumen diameter. Factors that may induce this response include passive adaptation to chronic hemodynamic stimuli, growth factors such as Ang II, and ROS. There are two kinds of remodeling: eutrophic or hypertrophic. In eutrophic remodeling the luminal diameter is reduced and the media becomes thicker with no overall change in cross-sectional area. However, in the hypertrophic scenario wall cross-sectional area is increased, cell size is increased, and extracellular matrix (ECM) accumulation of proteins such as collagen and fibronectin is enhanced (68-71). This latter type of remodeling is present in hypertension (72).

Findings in the literature suggest an important role of Nox-derived ROS in hypertrophic remodeling. New Zealand obese mice with metabolic syndrome have increased vascular $O_2^{\cdot-}$ and peroxynitrite production, Nox activity and adhesion molecule expression, leading to hypertrophic vascular remodeling in mesenteric resistance arteries characterized by increased media:lumen ratio and media cross-sectional area (73, 74). Systemic administration of gp91phox-dstat, an inhibitor of Nox2, decreases Ang II-induced vascular hypertrophy (75). Nox1 has also been implicated in the oxidative, pressor, and hypertrophic responses to Ang II (76, 77).

Vascular remodeling also requires degradation and reorganization of the ECM proteins. The most important enzymes involved in these spatial dynamics are matrix metalloproteinases, a group of zinc-dependent endopeptidases that degrade ECM proteins such as fibronectin and collagen. High expression/activation of MMPs is a critical component of the vascular remodeling process (78, 79). Several studies indicate a role of Nox-derived ROS in ECM turnover. ROS promote activation of MMP-2 and MMP-9 in VSMCs (80, 81). In addition, Ang II induces Nox-dependent expression of MMP-1, MMP-3 and MMP-9 (82). Mechanical stretch stimulates expression

and release of MMP-2 by Nox-derived ROS (83). Moreover, the chloride-proton antiporter chloride channel-3 (ClC-3), which is required for endosome-dependent signaling by Nox1 in VSMCs, contributes to neointimal hyperplasia after vascular injury by TNF α -mediated activation of MAPKs and MMP-9 (84-86). Similar effects of ROS on MMP-induced remodeling occur in other tissues as well, including TGF β -induced degradation of ECM and increased activity of MMP-9 during metastasis (71), TGF β -induced, Nox4-dependent fibrogenesis in lung injury (87), and TNF α -mediated, Nox-derived ROS-dependent expression of MMP-9, but not MMP-2, in cardiomyocytes (88).

Lipopolysaccharide (LPS) induces ROS generation and increases the expression of Nox1 and Nox2, as well as the expression of several MMPs. Recent work showed that the regulation of MMPs and migration in macrophages stimulated with LPS is dependent on Nox2-, but not Nox1-derived ROS. While Nox1 small interfering RNA (siRNA) did not inhibit LPS-mediated expression of MMPs, Nox2 siRNA inhibited the expression of MMP-9, 10 and 12 (89).

6. PHENOTYPIC MODULATION (PROLIFERATION AND DIFFERENTIATION)

ROS including O₂⁻ and H₂O₂ have important roles in the regulation of both proliferation and differentiation (39, 90). These mechanisms are crucial for not only the control of vasomotor tone, but also tissue repair and vascular remodeling. Low concentrations of O₂⁻ and H₂O₂ induce proliferation, moderate concentrations induce either cell stasis or hypertrophy, and very high concentrations can induce apoptosis (39, 91-95). Thus, as with other cellular functions, the amount of ROS produced, their source and the subcellular compartment in which they are generated greatly influence the final response.

Differentiated VSMCs express contractile proteins such as smooth muscle α -actin, calponin and smooth muscle myosin heavy chain, and have low proliferative capability and synthetic activity. In cardiovascular pathologies, VSMCs switch to the synthetic phenotype, proliferate and migrate, predisposing to vascular hypertrophy and lesion formation with elevated production of extracellular matrix (90). The mechanisms behind this phenotype switching are still very obscure; however, it is clear that physical and chemical changes in the environment modulate this process, namely humoral factors (platelet-derived growth factor (PDGF), TGF β , Ang II, endothelin-1 (ET-1)), neuronal signals, mechanical injury and changes in mechanical forces affecting cell-cell interaction and extracellular matrix (90). Nox1 and Nox4 appear to play very different roles during phenotypic modulation.

Nox4 is crucial for the maintenance of differentiation marker gene expression in VSMCs, stem cells, fibroblasts and pulmonary artery smooth muscle cells (14, 96-99). Low serum conditions or TGF β have been used as an external signal to induce differentiation in

VSMC. A recent study using TGF β suggests that Nox4 regulates smooth muscle α -actin gene expression via activation of p38MAPK and the serum-response factor (SRF)/myocardin-related transcription factor (MRTF) pathway in response to TGF β . Importantly, Nox4 has also been implicated in the differentiation of adipocytes, osteoblasts, chondrocytes and myofibroblasts. In adipocytes, Nox4 acts as a switch from insulin-induced proliferation to differentiation by controlling MAPK phosphatase-1 (MKP-1) expression, which controls ERK1/2 signaling (100). Similar to VSMCs, Nox4 mediates TGF β -stimulated fibroblast differentiation into a profibrotic, myofibroblast phenotype as well as the accompanying matrix production (101).

In contrast to the role of Nox4 in differentiation, Nox-mediated cell proliferation has been described in a several cell types present in blood vessels, kidney, liver, heart, lung epithelial cells and wide range of cancer cells (39, 102-105). In the blood vessel wall, ROS-mediated proliferation of VSMCs, adventitial fibroblasts and endothelial cells has been shown to be dependent on the Nox family proteins (105-107). Importantly, Nox isoforms seem to have different roles depending on their subcellular compartmentalization (8, 14).

Nox1 is fundamentally important in cell proliferation in VSMCs. Nox1 is located in caveolae and downregulation of caveolin decreased ROS production as well cell proliferation in VSMCs treated with Ang II (37, 84, 108, 109). In addition, it has been implicated in the growth response to PDGF, thrombin and ET-1. Overexpression of Nox1 potentiates the effect of Ang II on vascular hypertrophy while downregulation inhibits it (103, 110-112). The importance of Nox1 in proliferation has also been established in studies of neointima formation using genetically modified animal models (108), where injury-induced neointima formation was reduced in Nox1 deficient animals and proliferation and migration were reduced in VSMCs cultured from these animals. Conversely, overexpression of the Nox1 activator NoxA1 in mouse carotid arteries increased O₂⁻ production in VSMCs and enhanced neointimal formation and atherosclerotic lesion formation (113). The mechanism appears to involve thrombin-induced activation of redox-sensitive mitogenic protein kinases that lead to proliferation.

The molecular mechanisms controlling Nox1-mediated proliferation are only partially understood, but include activation of pro-growth signaling molecules such as kinases (p38MAPK and Akt), Ras and redox-sensitive transcriptional factors and inhibition of protein tyrosine phosphatases (112, 114). Only a few direct targets of ROS have been identified, and these are usually proteins with low pKa cysteine residues that can be oxidized by H₂O₂ (115, 116). Thus, antioxidant enzymes such as compartmentalized peroxiredoxins or thioredoxin are important regulators of the overall response (116).

Other recent findings underline the importance of cross-talk between redox pathways in Nox1-dependent

proliferation. Manipulating the extracellular oxidation-reduction state in aortic segments and VSMCs by altering the cysteine concentration (117) leads to an upregulation of Nox1 and an increase in proliferation. The mechanism appears to involve epidermal growth factor receptor phosphorylation by cleaved extracellular ligands following by ERK 1/2 activation.

In endothelial cells, Nox2 and Nox4 are the homologues that participate in cell proliferation. Their roles are not additive, but may be partially redundant (118-120). Both EaHy926 endothelial cells (a cell line) and human microvascular endothelial cells utilize Nox2 and Nox4 equally for ROS production and proliferation. Depletion of Nox1 in these cells does not alter ROS levels under basal conditions (120). Another study showed that overexpression of Nox4 enhanced receptor tyrosine kinase phosphorylation and the activation of ERK1/2, leading to enhanced proliferation and migration of endothelial cells (121).

Nox2 has also been described as an important regulator of fibroblast proliferation since its downregulation results in reduction of serum-induced proliferation (122, 123). Others findings suggest that ROS-derived from Nox family proteins in fibroblasts act as a paracrine stimulus on intimal cells and VSMCs to activate MAPKs and induce hypertrophy (124). Recently, it has been shown that Nox2 can regulate intracellular growth signaling pathways in adult hippocampal stem/progenitor cells, suggesting that, at least in the brain, ROS are important for maintaining specific cell populations and are not just related with deleterious conditions (125).

7. MIGRATION

Migration is a natural cellular process that is an integral part of vascular development as well as the healing response after vascular injury (126). ROS were first implicated in vascular migration more than 15 years ago when Sundarasan *et al* (104) showed that PDGF-induced VSMC migration was inhibited by catalase. Direct involvement of Nox-derived ROS in migration has also been shown for Ang II, ox-LDL, hypoxia, PDGF and vascular endothelial growth factor (VEGF) stimulation of either VSMCs or endothelial cells (108, 127-131). The signaling molecules so far identified as being regulated by ROS during migration are Src, RhoA, protein kinase C, phosphoinositide-3 (PI3) kinase, MAPKs including ERK1/2 and p38MAPK, NFkappaB, cofilin, slingshot-1 phosphatase (SSH1L) and Akt (126).

Several groups have described an interrelationship between PDGF and Nox1 or between basic fibroblast growth factor (bFGF) and Nox1 during migration (108, 127, 128, 132, 133). Other work showed that heme oxygenase (HO-1) and carbon monoxide (CO) inhibit PDGF stimulated VSMC migration via inhibition of Nox1 activity (134). The mechanisms mediating the role of Nox1 in migration include the modulation of cytoskeletal rearrangement as well as regulation of matrix metalloproteinases. In terms of cytoskeletal dynamics,

PDGF regulates SSH1L phosphatase, an enzyme responsible for activation of cofilin and actin depolymerization, through Nox1-derived ROS (135). Similarly, bFGF activates c-Jun N-terminal kinase (JNK) via Nox1-dependent ROS production (136). Nox1 siRNA blocks the up-regulation of MMP-9 in K-Ras-transformed normal rat kidney cells. Downregulation of Nox1 inhibits the activation of inhibitor of NFkappaB kinase-alpha (IKKalpha) and the degradation of IkappaBalpha, thus suppressing the NFkappaB activity in the promoter of MMP-9 (137). In other models of migration, Nox1 knockdown inhibits the directional persistence of migration via Rho-dependent modulation of integrin expression (138).

Nox4 also appears to have an important function in migration (139). Knockdown of Nox4 inhibits insulin-like growth factor-1 (IGF-1)-induced migration at least in part due to suppression of MMP2 and MMP9 induction (139). PDGF-induced migration is inhibited by siRNA against Nox4 or its regulator Poldip2 by a mechanism involving focal adhesion turnover (24). In a co-culture system of stromal mammary cells and epithelial cells, Nox4-dependent ROS production by TGFbeta stimulation of stromal cells mediates epithelial cell migration (140). Conversely, Nox4 overexpression has been shown to inhibit Ang II-induced myofibroblast migration via an H₂O₂ dependent pathway (141).

Normally, NO inhibits VSMC migration. However, in smooth muscle cells exposed to high glucose, NO loses its ability to inhibit migration due to glucose-induced oxidation of the sarco/endoplasmic reticulum ATPase (SERCA) (142). Both Nox1 and Nox4 are upregulated in these conditions, and knockdown of either oxidase restores the inhibition of migration by NO. Blocking activation of Rac1 or p47phox has the same effect. Thus, it appears that the Nox family is the source of the oxidants responsible for the failure of NO to inhibit SMC migration in high glucose conditions (142). Similar results have been described in cultured aortic SMC from obese Zucker rats, a model of obesity and insulin resistance. Upregulation of Nox4 by TGFbeta via Smad2 promotes oxidation of SERCA and consequently impairs NO-mediated inhibition of serum-induced VSMC migration (143).

8. APOPTOSIS AND AUTOPHAGY

Cell survival/growth and cell death are cellular responses integral to all physiological processes. However, in many pathological conditions cell death induces tissue dysfunction leading to disease. Cells from renewable tissues have two mechanisms to avoid uncontrolled growth, namely cellular senescence, which consists of permanent cell cycle arrest, or initiation of cell death programs (apoptosis or autophagy). Apoptosis is defined as programmed cell death while autophagy represents a lysosomal degradation pathway of cytoplasmic components such as protein aggregates and damaged organelles. The primary function of autophagy is to maintain cellular homeostasis. Cells already terminally differentiated, such

as neurons and cardiomyocytes, cannot become senescent (144, 145), but are subject to both apoptosis and autophagy.

ROS have been linked to cell death extensively in the literature. It is believed that temporally and spatially produced ROS regulate specific redox signaling pathways, while prolonged ROS generation in a diffuse manner can induce oxidative damage and consequently cell death (146, 147). Because Nox enzymes have specific subcellular localizations, they are likely to regulate the more specific death pathways. It is known, for example, that ROS regulate pro-apoptotic signaling (p38MAPK; JNK; apoptosis signal-regulating kinase (ASK1); Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII); C/EBP-homologous protein (CHOP)) as well as anti-apoptotic pathways (Akt, ERK1/2, and heat shock proteins (HSPs)) (146).

Apoptosis is induced in vascular endothelial cells by homocysteine, TNF α and remnant lipoprotein particles (148-150). In addition, UV radiation promotes activation of Nox with consequent increased apoptosis in keratinocytes (151). Studies in the literature have implicated Nox2 in cardiomyocyte apoptosis induced by Ang II. In the presence of apocynin or in p47phox knockout mice apoptosis is abolished, suggesting the involvement of Nox2 (Nox1 and 3 are not detected in these cells) (152). Nox4 also appears to induce apoptosis in cultured cardiomyocytes as well mouse heart tissue. Nox4-induced apoptosis is accompanied by cytochrome-c release and is reversed by Bcl-xl and manganese superoxide dismutase (MnSOD), suggesting involvement of the mitochondrial pathway. In addition, cardiac-specific Nox4 knockout mice have attenuated cardiomyocyte apoptosis (153, 154).

It is known that inactivation of the tumor suppressor p53 is strongly associated with pathological conditions in cardiovascular diseases. Overexpression of p53 promotes an increase in ROS in VSMCs with inhibition of proliferation and induction of apoptosis (102, 155-157), indicating that cell-cycle progression regulated by p53 in VSMCs is negatively regulated by ROS. Moreover, it has been described that VEGF-B is a potent negative regulator of apoptosis, potentially by inhibiting the expression of proapoptotic BH3-only proteins (members of the proapoptotic branch of the Bcl-2 proteins) in mouse primary VSMCs (158).

Another mechanism leading to apoptosis is endoplasmic reticulum (ER) stress. This is a type of cellular dysfunction in which ER function, especially protein synthesis, is compromised, generating a specific signaling response called the Unfolded Protein Response (UPR) in order to restore homeostasis (159, 160). Transient ER stress is part of normal cellular physiology; however, chronic ER stress contributes to pathological conditions such as atherosclerosis, cardiac hypertrophy and the response to ischemia. If UPR fails to restore ER function, ER-initiated apoptotic signaling is induced (161-163).

In recent years, an important convergence between oxidative stress and ER stress has been recognized (164). Initially, it was thought that oxidative stress resulted from ER stress upon redox folding of nascent proteins where ROS generation occurs during thiol-disulfide exchanges involving ER oxidoreductases, including the flavooxidase Ero1 and PDI (165). However, a range of studies has correlated ER stress with Nox family proteins (43, 166-173). In human VSMCs, Nox4 mediates apoptosis induced by 7-ketocholesterol. The mechanisms involved are related with the apoptotic branch of ER stress signaling, namely the CHOP signaling pathway and activation of inositol-requiring enzyme-1 (IRE1), a proximal sensor for the status of ER luminal protein folding during the UPR (172). Moreover, a recent study showed that proteasome inhibition during UPR can directly affect Nox4 activity and impairs VSMC viability during ER stress induced by tunicamycin (166). Another important role of Nox4 in ER stress is related to its functional interaction with the phosphatase PTP1B in the ER. PTP1B is involved in the IRE-1 branch of UPR signaling during ER stress (174). Oxidation of PTP1B by ROS-derived from Nox4 reduces its activity (175). Finally, a recent study demonstrated that ischemia/reperfusion injury induces Nox4 expression in renal tubule cells. The authors showed an interaction between Toll-like receptor 4, a 28 kDa form of Nox4 and the ER resident protein gp96, a homologue of cytosolic Hsp90 that is involved in ROS production and TNF receptor-associated factor 2 (TRAF2)/ASK1/JNK-mediated apoptosis in posthypoxic renal tubule cells. The authors suggested that Nox4 acts as a sensor in ER stress-induced proapoptotic signaling in this system (176).

Recent work showed that Nox2 also has an important role in oxidative and ER stress-induced apoptosis. Li *et al* (168) found that a branch of UPR signaling involving CHOP, ERO1 α and CAMKII induces Nox2 and oxidative stress, which are necessary for ER stress induced apoptosis. In *in vivo* experiments, Nox2 deficiency protects against ER stress-induced renal cell apoptosis and CHOP activation as well renal dysfunction.

ER stress and autophagy are completely inter-related processes (163). It has been described that ER stress induces autophagy to protect against cell death (163). In addition, in the last few years data have been presented that relate NADPH oxidases and autophagy (177). Expression of GRP78, an ER chaperone and a required component of autophagy in mammalian cells (178), is prevented by Nox4 siRNA treatment during ER stress in human VSMCs (172). Moreover, Nox4-derived H_2O_2 has been shown to activate Ras/ERK in endothelial cells, leading to induction of autophagy, prevention cell death and enhanced differentiation (171). Some studies have also related Nox2 to Toll-like receptor-activated autophagy in phagocytes (179).

9. HYPOXIA

A primary function of blood vessels is to provide nutrients and O_2 to maintain viability and physiological functions. Maintenance of oxygen homeostasis is crucial

for development and evolution (180). Chronic hypoxia is considered to be a major mechanism involved in aging as well chronic diseases like pulmonary arterial hypertension (PAH) and diabetes. During hypoxia (reduced O₂ availability) adaptive mechanisms are induced in the vasculature. Hypoxia-inducible transcriptional (HIF) factors are induced that regulate genes involved in energy metabolism, angiogenesis and apoptosis (181, 182). One such factor, HIF-1 α , is a major member of the HIF family and is normally degraded by the proteasome, but is stabilized in the presence of hypoxia (183). Interestingly, data in literature suggest that signaling induced by HIF is different among cell types. During development or regeneration processes, O₂ is required due to cellular proliferation and hypertrophy. In such conditions O₂ availability decreases and HIF-1 is activated. In response to tissue ischemia, vascular endothelial growth factor (VEGF), stem cell factor and PDGF are induced in a HIF-1-dependent manner (184, 185). These factors then promote recovery after hypoxic injury, as shown in the impaired recovery of function after femoral artery ligation in heterozygous HIF-1 α mice (185).

ROS have an important role in O₂ homeostasis in many studies and Nox family proteins are suggested to be involved. Nox2 appears to be an O₂ sensor in pulmonary airway chemoreceptors (186); however Nox2 is not required for the hypoxic pulmonary vasoconstriction response (187). Another study proposed that Nox1 is involved in induction of HIF-1, because Nox1-transfected cells show increased expression of HIF-1 that is inhibited by catalase and or DPI (188). In contrast, Nox4 is proposed to be a target of HIF-1 since overexpression of HIF-1 increases Nox4 expression and its downregulation prevents such an increase (189). Mutation of a putative hypoxia-responsive element in the Nox4 promoter abolishes its induction by HIF-1. Nox4 has also been suggested to act as an oxygen sensor in conjunction with the TWIK-related acid-sensitive K⁺ channel TASK-1 (190, 191).

10. PULMONARY HYPERTENSION

Chronic PAH is an important clinical condition present in wide range of cardiovascular patients. The development of pulmonary hypertension is characterized by chronic hypoxia, endothelial injury and subsequent inflammation (192). The pulmonary circulation responds to local airway hypoxia with vasoconstriction in order to direct blood flow to better-ventilated regions of the lung. However, chronic hypoxia causes vascular remodeling associated with a loss of relaxation to endogenous NO, increased levels of O₂⁻ and consequently increased pulmonary arterial pressure (193). As expected, ROS are involved in acute hypoxic vasoconstriction in the lung, as demonstrated by attenuation of vasoconstriction in the presence of SOD (194). Overexpression of extracellular SOD also attenuates vascular remodeling in chronic hypoxia (195).

In terms of the source of ROS involved in this response, many investigators have reported a role for NADPH oxidases. For example, Nox1 is upregulated in

pulmonary resistance arteries of newborn piglets exposed to long-term hypoxia (196). Nox2-deficient mice show impairment of the vasoconstrictor response to ET-1 in the pulmonary vasculature after exposure to chronic hypoxia (197). Moreover, p47phox-deficient mice exhibit a reduction of acute hypoxic pulmonary vasoconstriction, while non-hypoxia-induced vasoconstrictions are not affected (198). Disruption of the Nox2 gene also has chronic effects: it inhibits hypoxia-induced PAH and vascular remodeling (199, 200). A number of other studies, however, suggest that it is Nox4 that has an important role in the vascular remodeling process. Nox4 expression, but not that of the other Nox proteins, is upregulated in pulmonary artery smooth muscle cells (PASMC) of mice subjected to hypoxic conditions (201). This upregulation occurs via autocrine production of TGF β and insulin-like growth factor binding protein-3, and is necessary for PASMC proliferation (98). Another study found that downregulation of Nox4, but not of Nox2, promotes attenuation of hypoxic pulmonary vasoconstriction in bovine pulmonary arteries (202). The mechanisms by which hypoxia induces Nox4 and by which Nox4 influences vasoconstriction and remodeling are somewhat unclear, but likely involve the HIF family proteins and proliferator-activated receptor- γ (PPAR γ). ROS-derived from Nox4 increase the activity of HIF-1 (203) and HIF-2 α in PASMC (204). Diebold *et al* (189) found that hypoxia rapidly increases Nox4 mRNA and protein levels in PASMC as well as pulmonary vessels from mice in a HIF-1 dependent manner. PPAR γ appears to regulate hypoxia-stimulated Nox4 induction through an NF κ B-mediated mechanism in human PASMC (205). It is clear that the contribution of NADPH oxidases to pulmonary hypertension is complex, and that more work is needed to fully understand the mechanisms involved.

11. ATHEROSCLEROSIS

Atherosclerosis is a long-term inflammatory disease considered to be a public health issue worldwide. The disease consists of formation of atherosclerotic plaques in hemodynamically unstable regions of the vessel. Risk factors associated with disease development and progression are diverse, but include genetic propensity, obesity, hypertension, hypercholesterolemia, diabetes and smoking. Many of the steps of the progression of disease are ROS-sensitive. Plaque development depends on the subendothelial retention of lipids; cytokines that induce inflammatory responses including endothelial activation and recruitment of immune cells (macrophages, dendritic cells, neutrophils, B and T cells); cell proliferation, migration, and secretion of extracellular matrix proteins; and cell death and activation of coagulation factors (206). All these processes have all been associated with activation of Nox family proteins.

Early in disease development, macrophages and other cells of the immune system that are rich in Nox2 invade the vessel wall, and induction and activation of other Nox proteins occur in resident vascular cells (39). ROS-derived from Nox enzymes contribute to macrophage-

mediated oxidation of LDL, which can then activate Nox proteins in vascular cells, resulting in expression of adhesion molecules and the recruitment of immune cells (207, 208). It has been shown that p47phox is required for atherosclerotic plaque evolution in ApoE transgenic mice (209, 210). Other data also support a role for Nox family proteins in atherosclerosis, such as the observation that Nox1 mRNA and Nox4 mRNA in nonphagocytic cells, as well as expression of p22phox in phagocytes, are increased in atherosclerotic human coronary arteries (39, 130, 211, 212). In addition, recent work showed that knockout of Nox1 in mice lacking the ApoE gene also reduces lesion formation (211).

In the last few years, the relevance of ER stress and autophagy to the progression of atherosclerotic plaques has begun to be appreciated (213-215). Human atherosclerotic plaques show increased ER stress as assessed by expression of GRP78 and the pro-apoptotic factor CHOP. In the same study, the authors found 7-Ketocholesterol (cited above as an inducer of Nox4) in the fibrous caps of atherosclerotic plaques. Treatment of cultured coronary artery smooth muscle cells with 7-Ketocholesterol induced the upregulation of chaperones and apoptosis, and this effect was abolished by antioxidants (172). Apoptosis of macrophages regulated by CHOP, and as a consequence an increase of the instability atherosclerotic plaques, has also been described by different groups (172, 216). More specifically, Thorp *et al* (217) tested the effect of CHOP deficiency *in vivo* by measuring aortic root lesions of fat-fed Chop^{+/+}ApoE^{-/-} and Chop^{-/-}ApoE^{-/-} mice. They found that plaque necrosis was reduced by approximately 50% and lesional apoptosis by 35% in the CHOP-deficient mice.

As part of the evolution of atherosclerosis, chronic inflammatory processes become associated with Toll-like receptors (TLRs), regulators of innate immunity (218). It has been demonstrated that TLRs can promote activation of Nox proteins. Stimulation of TLR4 with LPS induces ROS generation and NFkappaB activation in HEK293 cells via a mechanism involving Nox4 (219). Nox4-dependent ROS generation is also important for generation of proinflammatory cytokines by LPS in endothelial cells (220). Recently, palmitate was proposed to induce inflammation in human endothelial cells. Like LPS, palmitate increases O₂⁻ production and stimulates NFkappaB signaling via TLR4-mediated activation of Nox4 (221). Finally, high-fat feeding increases expression of Nox4 and bone morphogenic protein (BMP4) in thoracic aorta in wild type, but not TLR4^{-/-} mice (221).

12. ANGIOGENESIS

Angiogenesis consists of the formation of new capillaries from preexisting vessels and is part of the process of neovascularization. During angiogenesis, endothelial cells proliferate, migrate and form capillary tubes. As described above, ROS play a crucial role in all of these processes. Importantly, Nox family proteins are activated by angiogenic stimuli such as VEGF, Ang II, hypoxia, cytokines and shear stress (34, 222). Transgenic mice overexpressing p22phox have increased VEGF

expression and enhanced angiogenesis in experimental atheromas (223). Rac-1 mediated O₂⁻ production mediates VEGF-induced placental angiogenesis (224) as well as that induced by overexpression of phosphodiesterase-2 (225). More specifically, a wide range of studies suggests a role for Nox2 in angiogenesis (226, 227). Studies using the hindlimb ischemia model in mice showed that Nox2 expression and ROS production are increased in bone marrow mononuclear cells (228). However, mice lacking Nox2 have impaired ischemia-induced blood flow recovery and neovascularization, which is associated with reduction of ROS production in bone marrow-derived cells as well as with a decrease in the number of endothelial progenitor cells in peripheral blood (228). Another study exposed p47phox deficient mice to arsenite in the drinking water. Environmental arsenic is a risk factor for developing vascular disease marked by pathologic remodeling. p47phox deficient mice were protected from arsenite-induced capillarization. *Ex vivo* arsenic exposure of endothelial cells led to increased O₂⁻ generation that was inhibited by downregulation of Nox2, suggesting that Nox2 is required for capillarization (229).

Nox2 is not the only Nox involved, however: Nox1 overexpression can induce expression of VEGF and its receptors and upregulate matrix metalloproteinase activity through ROS generation, thus promoting angiogenesis (230). Nox1 downregulation decreases endothelial migration and tube-like structure formation by a mechanism involving NFkappaB signaling (137). Notably, mice deficient in Nox1, but not Nox4, exhibit impaired tumor angiogenesis (231). The role of Nox4 may be context dependent, however. Nox4-null animals exposed to chronic pressure overload develop contractile dysfunction, hypertrophy, and cardiac dilatation, whereas Nox4-transgenic mice are protected. The mechanism appears to be related to Nox4-dependent preservation of myocardial capillary density after pressure overload. Overexpression of Nox5 induces endothelial proliferation and formation of capillary-like structures (22).

Alternatively, excess ROS production can impair angiogenesis. An example of this involves the effects of Ang II in formation of new vessels after ischemia. Ang II signaling has different effects on angiogenesis depending on the basal vascular oxidative stress. Elevated levels of basal ROS make the effect of Ang II on collateral growth inhibitory, while Ang II administered when basal levels of ROS are low promotes collateral growth (232). Another case where excess ROS have been shown to be inhibitory is in pulmonary artery endothelial cells from fetal lambs with persistent pulmonary hypertension. These cells have increased expression of Nox subunits (Nox4, Nox2, Rac1, p47phox and p67phox) and increased Nox activity, but exhibit decreased tube formation, cell proliferation, scratch recovery and cell invasion, suggesting that increased oxidative stress from Nox activity contributes to impaired angiogenesis in this model (233).

13. DIABETIC VASCULAR DISEASE

Diabetes mellitus is accompanied by an increased risk of developing cardiovascular disease. A wide range of

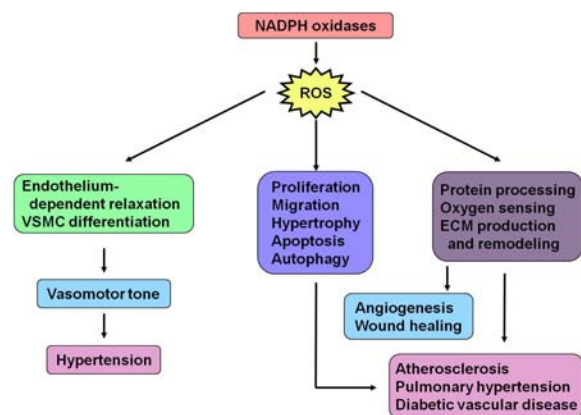


Figure 1. Diverse roles of NADPH oxidases in vascular physiology and pathophysiology. ROS generation by Nox enzymes maintain vascular tone and integrity, participate in the response to injury, and contribute to vascular diseases.

studies has implicated oxidative stress in the pathology of diabetes. Elevated oxidative stress is detected in diabetic patients and in animal models of diabetes (obese ob/ob mice, Zucker fatty rats, streptozotocin-induced diabetes) (234).

Hyperglycemia, hyperinsulinemia, and increased levels of lipids promote ROS generation in diverse cell types. The enzymatic sources of ROS include Nox family proteins, mitochondria and uncoupled NO synthases, with a very strong probability of cross-talk between them. Enhanced expression of p22phox, increased Nox activity and elevated $O_2^{\cdot -}$ production were found in mouse microvascular endothelial cells exposed to high glucose (235). High glucose, advanced glycation endproducts and free fatty acids induce expression and activity of Nox in VSMCs as well (236-239). In one study, downregulation of p47phox in VSMCs decreased $O_2^{\cdot -}$ generation (235), and no apparent role for eNOS, mitochondria or xanthine oxidase was found (240). In contrast, another group showed that ROS generation by hyperglycemia was decreased by an inhibitor of electron transport chain complex II, uncoupling protein-1 or MnSOD in aortic endothelial cells (241). In aortas of streptozotocin diabetic rats, uncoupled eNOS and Nox2 have been implicated in ROS generation (242). It is thus clear that there are multiple sources of ROS in the diabetic vasculature, depending on cell type, stimulus and diabetic model.

PKC appears to be extremely important in Nox regulation and activation in diabetes. For example, activation of Nox was abolished in diabetic PKC β knockout mice (243). Other isoforms of PKC have been implicated as well: PKC α mediates Nox activation in the kidney of diabetic rats, PKC δ in adipocytes (244) and PKC ζ in mesangial cells treated with high glucose (245). JNK activation and Akt inhibition may be downstream of Nox2-derived ROS, at least in beta-cells exposed to high glucose (246).

Insulin increases ROS generation in VSMCs, adipocytes, fibroblasts and macrophages (247-250). In macrophages, ROS induced by insulin are derived from Nox2

(250). In other insulin-sensitive cells, Nox4 appears to have important role in the response to insulin since its downregulation prevents ROS production and inactivation of phosphatase PTP1B, a widely expressed phosphatase implicated in the negative regulation of insulin signaling (251). Another study suggests that the upregulation of Nox4 by TGF β in VSMCs from obese Zucker rats is responsible for the impaired response to NO by a mechanism involving the oxidation of SERCA C674. Such mechanisms could explain the enhanced VSMC migration and neointima formation in type 2 diabetes (252).

Another effect of excess ROS generation in diabetes is impairment of acetylcholine-induced endothelium-dependent relaxation, a response that has been attributed to NADPH oxidases, at least in OLETF rats (253). Downregulation of Nox enzymes has also been shown to decrease the proinflammatory effects of AGEs in different cell types. For example, macrophages from Nox2 $^{-/-}$ mice showed inhibition of AGE effects (254). Downregulation of Nox1 impairs AGE-induced ROS production and induction of iNOS in VSMCs (237). Finally, treatment of db/db mice (a model of type 2 diabetes) with the $O_2^{\cdot -}$ scavenger tempol reduces expression of inflammatory genes in the vessel wall (236).

Pathways that inhibit Nox-dependent ROS production in diabetes are thus quite promising therapeutic targets for diabetic vascular dysfunction. One mechanism that reduces the enhanced PDGF-induced ROS generation that occurs in response to high glucose in VSMC is activation of the protein tyrosine phosphatase PTP ϵ . PTP ϵ negatively regulates PDGF β -receptor signaling, DNA synthesis and migration by reducing the phosphorylation level of the PDGF β receptor with subsequent downregulation of Nox1 and suppression of H_2O_2 generation (255). Another inhibitor of diabetes-induced oxidative stress is resveratrol. Resveratrol is a natural phytophenol found in wine that exhibits cardioprotective effects. It has been shown to reduce oxidative/nitrative stress and improve NO availability by inhibition of TNF α -induced NF- κ B activation and expression and activation of Nox2 and iNOS in type 2 diabetes (256).

14. PERSPECTIVE

Studies summarized in this review make it clear that Nox-derived ROS play a role in virtually all aspects of vascular function, both physiologically and pathophysiologically (Figure 1). Individual Nox enzymes play distinct roles both within a cell and between cell types. Moreover, Nox proteins exhibit considerable cross-talk with other oxidant-generating systems (33). As we begin to understand this differential regulation of ROS-dependent signaling, we will be able to more rationally develop therapies targeted to specific sources of ROS, rather than relying on global antioxidant treatments that have been suboptimal in the past. The complexities of redox regulation of vascular function provide fertile ground for future investigation.

15. ACKNOWLEDGMENTS

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Abbreviations: The abbreviations used are: Ang II, angiotensin II; ApoE, Apolipoprotein E; ASK1, apoptosis signal-regulating kinase; bFGF, basic fibroblast growth factor; BH₄, tetrahydrobiopterin; CaMKII, Ca²⁺/calmodulin-dependent protein kinase II; CHOP, C/EBP-homologous protein; CO, carbon monoxide; ECM, extracellular matrix; EDHF, endothelium-dependent hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; ERK1/2, extracellular signal regulated kinase; ERO-1, ER oxidoreductin 1; ET-1, endothelin-1; H₂O₂, hydrogen peroxide; HIF, Hypoxia-inducible factor; HO-1, heme oxygenase; HSP, heat shock protein; IGF-1, insulin-like growth factor-1; IKK α –inhibitor of NF κ B kinase- α ; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein

kinase; MKP-1, MAPK phosphatase-1; MMP, matrix metalloproteinase; MnSOD, manganese superoxide dismutase; MRTF, myocardin-related transcription factor; NF κ B, nuclear factor kappa B; NO, nitric oxide; NOX, NADPH oxidase; O₂⁻, superoxide anion; OLETF, Otsuka Long-Evans Tokushima Fatty; PAH, pulmonary arterial hypertension; PASMC, pulmonary artery smooth muscle cells; PDGF, platelet-derived growth factor; PDI, protein disulfide isomerase; PI3K, phosphoinositide-3 kinase; PKC, protein kinase C; PPAR γ , peroxisome proliferator-activated receptor- γ ; PTP, tyrosine-protein phosphatase; ROS, reactive oxygen species; SERCA, sarco/endoplasmic reticulum ATPase; SHP2, SH2 domain-containing protein tyrosine phosphatase-2; SRF, serum-response factor; SSH1L, slingshot-1 phosphatase; TASK1, Tandem-pore acid-sensing potassium channel-1; TGF β , transforming growth factor beta; TLR, toll-like receptors; TNF α -tumor necrosis factor alpha; TRAF2, TNF receptor-associated factor 2; UPR, unfolded protein response; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cells.

Key Words: Reactive oxygen species, NADPH oxidases, Nox, Vascular disease, Review

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