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Review

Functional Heterogeneity of Nadph Oxidases in Atherosclerotic and Aneurysmal Diseases

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NADPH oxidases (NOX) are enzymes that catalyze the production of reactive oxygen species (ROS). Four species of NOX catalytic homologs (NOX1, NOX2, NOX4, and NOX5) are reportedly expressed in vascular tissues. The pro-atherogenic roles of NOX1, NOX2, and their organizer protein $p47^{phox}$ were manifested, and it was noted that the hydrogen peroxide-generating enzyme NOX4 possesses atheroprotective effects. Loss of NOX1 or $p47^{phox}$ appears to ameliorate murine aortic dissection and subsequent aneurysmal diseases; in contrast, the ablation of NOX2 exacerbates the aneurysmal diseases. It is possible that the loss of NOX2 activates inflammatory cascades in macrophages in the lesions. Roles of NOX5 in vascular functions are currently undetermined, owing to the absence of this enzyme in rodents and the limitation of the experimental procedure. Thus, it is possible that the NOX family of enzymes exhibits heterogeneity in the atherosclerotic diseases. In this aspect, subtype-selective NOX inhibitor may be promising when NOX systems serve as a molecular target for atherosclerotic and aneurysmal diseases.

Key words: Nadph oxidase, Atherosclerosis, Abdominal aortic aneurysm, Aortic dissection

Introduction

NADPH oxidases (NOX) are enzymes that transfer electrons across biological membranes from NADPH. In general, the electron acceptor is oxygen, and the product of the enzymatic reaction is superoxide ($O_2^{\bullet -}$); thus, activation of NOX enzymes leads to generation of reactive oxygen species (ROS)[1-3]. Following the cloning of the gene that encodes NOX2 (*Cybb*)[4, 5], the role of NOX systems in phagocytic and host defense functions including the respiratory burst was investigated[1, 6]. Non-phagocyte NOX homologs were subsequently identified and were functionally characterized in other cell types, including the cells that make up vascular components[7]. Despite the host defense functions of NOX2, numerous lines of research indicate that dysregulation of NOX isozymes, including NOX2, potentiates oxidative stress in inflamed lesions and exerts deleterious roles in a variety of inflammatory diseases[1, 3, 7].

Atherosclerosis, a chronic vascular disease characterized by functional and structural disorders in whole arterial trees, can be a cause of lethal cardiovascular events, such as myocardial infarction and stroke[8]. Pharmacotherapy with statins has been shown to reduce cardiovascular event rates significantly in randomized, placebo-controlled clinical trials in some cases; however, statins are not always sufficient for reducing the events[9]. For example, recurrent ischemic events following acute coronary syndrome occur in >20% of patients by 36 months after the primary events despite optimal cholesterol lowering therapy and medical care[10]. Thus, the molecules expressed in the vascular walls, which function as critical factors for direct control of atherosclerotic cardiovascular diseases, should be targeted to achieve better therapeutic outcomes. It is believed that ROS production in vascular walls has a pivotal role in atherogenesis; thus, the behavior and impact of NOX isozymes in atherogenesis have been extensively studied.

Abdominal aortic aneurysm (AAA), one of the representative atherosclerotic diseases, is a major cause of cardiovascular deaths and the tenth leading cause of death in men aged over 65 years in western coun-

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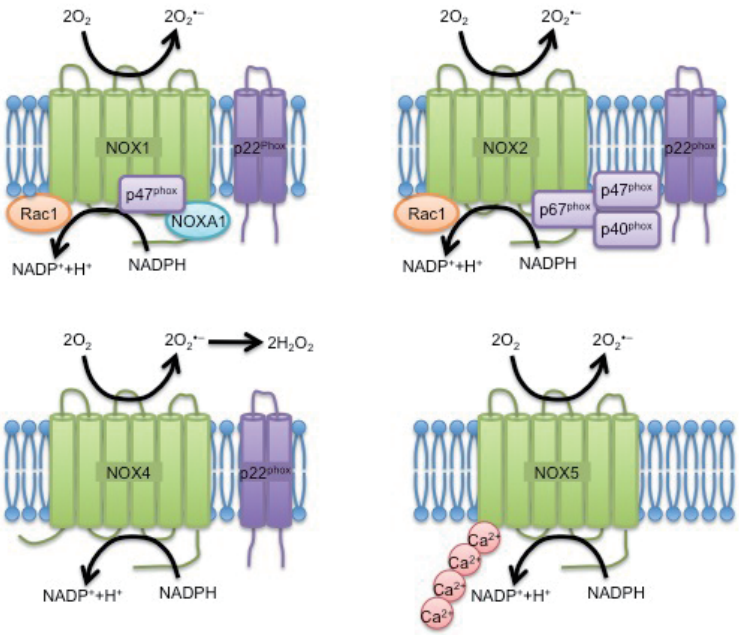

Fig. 1. Structure and composition of vascular NOX systems

tries[11]. The prevalence of AAA in the elderly has been estimated at 2.2% in a recent Swedish study[12] and at 4% to 8% in earlier studies in the USA, Australia, Denmark, and UK[13-16], with a mortality rate of 80% following AAA rupture[17]. Although AAA rupture is life threatening, molecular targets for this disease have not yet been identified. In turn, AAA is often treated surgically, with artificial vessels or stent grafts[18]. To reduce surgical exposure and its accompanying complications, various candidate molecules targeting AAA, including NOX isozymes, are currently undergoing experimental trials. In this brief review, we summarize the roles of NOX family enzymes in the regulation of vascular functions as well as the pathogenesis of atherosclerosis and AAA and discuss their functional heterogeneity.

NOX Family Enzymes and Vascular Function

NOX systems are composed of catalytic subunits (e.g., NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2), a smaller membrane-bound protein that stabilizes the NOX subunit within membranes ($p22^{phox}$), and up to three cytosolic regulatory subunits including an organizer protein ($p47^{phox}$ or NOXO1), activator proteins ($p67^{phox}$ or NOXA1), and small GTPases (Rac1 or Rac2)[1]. These systems stimulate the production of ROS, including $O_2^{\bullet -}$ and hydrogen peroxide (H_2O_2)[3]. Since $O_2^{\bullet -}$ is hardly permeable to biological membranes and is rapidly converted to other ROS, its working range is mostly local. Accordingly, depending on the subcellular localization of NOX, $O_2^{\bullet -}$ is released either intracellularly or extracellularly, eliciting corresponding internal signaling or paracrine effects. Superoxide dismutase (SOD) rapidly converts $O_2^{\bullet -}$ to longer-lasting and membrane-permeable H_2O_2, leading to the distinct signal transduction and expanded range of action. Among seven species of NOX catalytic homologs, four of which (NOX1, NOX2, NOX4, and NOX5) are reportedly expressed in vascular tissues. For further details regarding the molecular basis of the NOX family, please refer the previous review articles[1-3]. Distinct roles of NOX isozymes in vascular functions are summarized below.

NOX1

Nox1 is expressed in vascular endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and adventitial fibroblasts at the plasma membrane, caveolae, and endosomes, and associates with $p22^{phox}$, $p47^{phox}$, Rac GTPases, and NOXA1[2, 3] (**Fig. 1**). In VSMCs, NOX1 is reportedly inducible and activatable by certain vasoactive agents as well as growth factors, including angiotensin II (AngII)[2], basic fibroblast growth factor (bFGF)[19], and platelet-derived growth factor (PDGF)[20]. Furthermore, NOX1 is inducible by the pro-atherogenic cytokines IFN-γ, which is mediated through Janus kinase/signal transducer and activator of transcription (JAK/STAT)-mediated transcriptional regulations[21]. Accordingly, dysregulation of this enzyme in VSMCs appears to

cause vascular disorders *in vivo*, such as hypertension[22, 23], vascular hypertrophy[24], and vascular inflammation[25, 26], whereas its physiological functions remain largely elusive.

NOX2

NOX2, a superoxide generating enzyme, is expressed in every type of vascular component cells, except VSMCs, in large arteries, and associates with $p22^{phox}$, $p47^{phox}$, Rac GTPases, and $p67^{phox}$ (**Fig. 1**)[2, 3]. ECs and adventitial fibroblasts are considered the primary sources of NOX2-derived superoxide, even in large arteries[10]. NOX2 can be activated by pathways similar to NOX1, such as phosphorylation of $p47^{phox}$ by PKC and Src[27, 28]. *In vitro* experiments have shown that NOX2 is activable by the multiple classes of inflammatory cytokines, including IL-17[29] and IFN-γ[30]. Importantly, it was noted that NOX2-generated $O_2^{\bullet -}$ induces vascular constriction, since $O_2^{\bullet -}$ rapidly reacts with and neutralizes nitric oxide ($NO^{\bullet}$), a robust EC-derived vasodilator. Indeed, NOX2 expression levels were inversely correlated with EC-dependent vasodilation in isolated murine aortas[31]. The over-activation of NOX2 in vascular tissues may associate with vascular diseases, such as hypertension[2, 32-34] and pathological angiogenesis[35].

NOX4

NOX4 is expressed in every type of resident cells in the vasculature[2, 3] and is distributed intracellularly in the perinuclear space or the endoplasmic reticulum in ECs[36, 37], the nucleus in ECs[38], and in focal adhesions and stress fibers in VSMCs[39]. NOX4 is thought to be constitutively active[40], since NOX4 associates with $p22^{phox}$, but not with the stress response molecules $p47^{phox}$ or Rac GTPases, unlike NOX1 and NOX2 (**Fig. 1**). Accordingly, NOX4 ensures the baseline production of ROS, particularly H_2O_2. Earlier investigations identified transforming growth factor-β as an activator of NOX4 signals[41]. Cell-based experiments found that NOX4 mediates proliferation in pulmonary VSMCs[42, 43]. Furthermore, NOX4 elicits mitotic and anti-apoptotic actions in ECs[44-46]. Contrarily, presence of NOX4 appears to be necessary for maintaining VSMCs in the non-proliferative quiescent state and mediates TNF-α-induced apoptosis in ECs[28, 47, 48]. In addition to mitotic actions, roles of NOX4 in cell motility have been described. Indeed, silencing of *Nox4* by siRNA reportedly impairs PDGF-induced migration in VSMCs[49] and wound closure in ECs[46]. However, it is also noted that *Nox4* over-expression in VSMCs[49] or AngII-stimulated adventitial fibroblasts[50] decelerates cellular motility. Such opposite phenotypes regarding mitosis and kinetics are still controversial and presumably depend on the cell types or unidentified environmental conditions.

A gene-targeting study showed that *Nox4* deficiency delays recovery of blood flow in a femoral artery ligation model in mice, which is due to the reduction of angiogenic responses in ECs[51]. Furthermore, *Nox4* deficiency in AngII-administrated mice suppressed $NO^{\bullet}$ production and EC-dependent vasodilation and facilitated aortic wall thickening. Thus, it is likely that NOX4 and its product H_2O_2 in ECs have roles in vascular homeostasis even under the inflammatory conditions.

NOX5

NOX5 is structurally distinct from other NOX enzymes, since this isozyme contains an additional regulatory domain in its N-terminal. Unlike other NOX isozymes, additional subunits are unnecessary for NOX5 activity; alternatively, this isozyme is regulated by calcium binding to N-terminal EF-hands (**Fig. 1**). NOX5 is detectable intracellularly in the cytoskeletal fraction, endoplasmic reticulum, and plasma membrane[52-54]. Since NOX5 is not expressed in rodents, this molecule has been analyzed mainly utilizing cultured cells and isolated tissues. It was reported that NOX5 participates in PDGF-induced proliferation through the JAK/STAT signals in VSMCs[55]. Furthermore, forced expression of NOX5 in ECs facilitates proliferation and angiogenic tube formations[52]. Contrarily, adenoviral-mediated transduction of NOX5 in isolated aortas potentiates production of $O_2^{\bullet -}$, thereby accelerating consumption of EC-derived $NO^{\bullet}$. Accordingly, the forced expression of NOX5 in aortas impairs EC-dependent relaxation, and potentiated phenylephrine-induced contraction[56]. These results imply the potential regulatory roles of NOX5 in vascular function; however, the physiological and pathophysiological significance of endogenous NOX5 is currently unclear.

Roles of NOX Family in Atherosclerotic and Aneurysmal Diseases

Atherosclerosis

Accumulating evidence showed that ROS-mediated oxidative stress in atherosclerotic lesions induces various inflammatory elements, such as endothelial adhesion molecules (e.g., ICAM-1, VCAM-1, E-selectin) and inflammatory cytokines and chemokines (e.g., IFN-γ, IL-1β, IL-6, MCP-1), through redox-sensitive transcription factors (e.g., NF-κB and AP-1). Overexpression of adhesion molecules in ECs facilitates

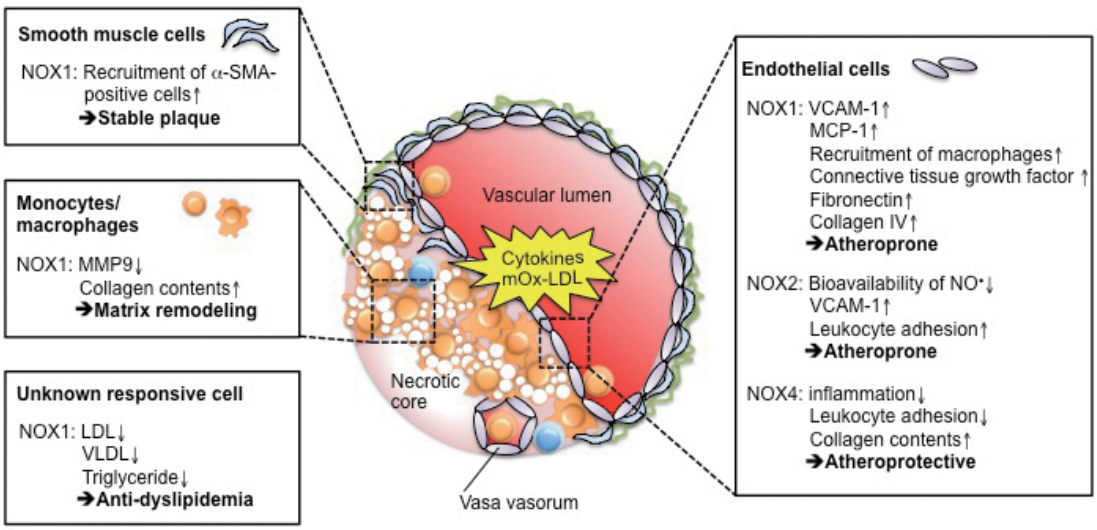

Fig. 2. Roles of vascular NOX systems in atherosclerosis

NOX1 and NOX2 play a leading role in the development of atherosclerotic lesions. NOX1 participates in the induction of VCAM-1 and MCP-1 in vascular endothelial cells, thereby accelerating the recruitment of macrophages into the lesions, while loss of NOX1 facilitates MMP9 secretion and reduces the amount of fibronectin and collagen IV in the lesions. In addition to those actions, NOX1 appears to associate with the recruitment of α-SMA-positive cells in lesions and potentially antagonizes plasma dyslipidemia. Excess production of $O_2^{\bullet -}$ by NOX2 in vascular endothelial cells reduces the bioavailability of NO• and facilitates VCAM-1-mediated adhesion of leukocytes in the vascular lumen, resulting in the acceleration of atherosclerotic development. NOX4 in vascular endothelial cells potentially antagonizes inflammatory responses, leading to the reduction of atherosclerotic lesions. α-SMA: α-smooth muscle actin, MMP: matrix metalloproteinase.

leukocyte adhesion to ECs, and MCP-1 potentiates cellular motility in monocytes/macrophages, thereby accelerating infiltration of those cells into the lesions. Pro-inflammatory cytokines mediate immune responses in immunocompetent cells, including T-cells and mast cells, in the lesions[57]. It is believed that excess activity of NOX family enzymes triggers such inflammatory responses in atherosclerotic lesions.

Sorescu *et al.* investigated the distribution of oxidative stress and NOX family in human atherosclerotic plaques[58]. Whereas oxidative stress was present homogenously throughout the intima, media, and adventitia in nonatherosclerotic coronary arteries, additional intense areas of oxidative stress were detectable in the shoulders of coronary plaques, which are enriched in macrophages and α-actin-positive cells. $p22^{phox}$ and NOX2 were co-expressed mainly in macrophages, whereas NOX4 was found only in nonphagocytic vascular cells. Expression of NOX2 and $p22^{phox}$ mRNA was associated with the severity of atherosclerosis. NOX2 expression correlated with the plaque macrophage content while NOX4 expression correlated with the content of α-actin-positive cells. NOX1 expression was marginal both in human coronary arteries and in isolated vascular cells.

Contribution of endogenous NOX family members to atherosclerosis, except for NOX5, has been mainly investigated by using gene-targeting techniques and atherosclerosis-prone mice, *Apoe*-null mice and *Ldlr*-null mice (**Fig. 2** and **Table 1**). Barry-Lane *et al.* demonstrated that ablation of $p47^{phox}$ a common organizer protein between NOX1 and NOX2 in vascular tissues, suppresses atherosclerotic lesions in *Apoe*-null mice[59]. Gray *et al.* noted that atherosclerosis in *Apoe*-null mice is aggravated by streptozotocin-induced diabetes, which is ameliorated by *Nox1* deficiency or GKT137831, a specific orally active NOX inhibitor with a specificity for Nox1 and Nox4[60]. Amelioration of atherosclerosis by *Nox1* deficiency was accompanied by the reduction of MCP-1 and VCAM-1 expression, thereby decreasing recruitment of macrophages. Furthermore, NOX2 is reportedly enriched in ECs and macrophages in the atherosclerotic lesions in *Apoe*-null mice, and generates excessive $O_2^{\bullet -}$, leading to reduction of bioavailability of NO•[61]. Since NO• exerts potent atheroprotective effects[62], deficiency of *Cybb* ameliorates atherosclerotic diseases in mice. In contrast, EC-specific transduction of *Cybb* accelerates macrophage recruitment to the atherosclerotic lesion in *Apoe*-null mice, whereas it does not affect the development of lesions[63]. Considering the predominance of inflammatory cytokines

Table 1. NOX genotype and atherogenesis in mice

Isozyme (gene symbol)	Genotype and diet/treatment	Atherogenesis	Other phenotype	Ref.
NOX1 (*Nox1*)	*Nox1*$^{-/y}$*Apoe*$^{-/-}$ Streptozotocin, i.p.	↓ ↓ ↓	Aortic ROS ↓ Macrophage recruitment ↓ Aortic leukocyte adhesion ↓ Aortic VCAM-1 ↓ Aortic MCP-1 ↓ Aortic connective tissue growth factor ↓ Aortic fibronectin ↓ Aortic collagen Ⅳ ↓	60
	Nox1$^{-/y}$*Apoe*$^{-/-}$ Western diet (21% fat and 0.15% cholesterol)	Unchanged	Plasma cholesterol ↑ Plasma LDL/VLDL ↑ Plasma triglycerides ↑ Aortic ROS ↑ Aortic collagen contents ↓ Aortic α-SMA positive cells ↓ Aortic MMP-9 expression ↓	66
NOX2 (*Cybb*)	*Cybb*$^{-/y}$*Apoe*$^{-/-}$ Western diet (21% fat and 0.15% cholesterol)	↓ ↓	Aortic ROS ↓ L-NAME-induced contraction ↑	61
	CYBB-Tg *Apoe*$^{-/-}$ Chow diet AngⅡ infusion	Unchanged	Aortic ROS ↑ Aortic VCAM-1 ↑ Macropahge adhesion to aorta ↑	63
NOX4 (*Nox4*)	*Nox4*$^{flox/flox}$-*Cre-ERT2*$^{+/0}$*Apoe*$^{-/-}$ Western- type diet (42% fat, 0.15% cholesterol)	↑ ↑ ↑	H_2O_2 production ↓ Aortic collagen contents ↓ Macrophage recruitment ↑ Leukocyte adhesion to ECs ↑	67
p47^{phox} (*Ncf1*)	*Ncf1*$^{-/-}$*Apoe*$^{-/-}$ High-fat diet (15% fat)	↓ ↓ ↓	ROS in VSMCs ↓	59

in atherosclerotic lesions[57], it is suspected that the NOX system in the lesions is potentiated by pro-atherogenic cytokines, such as IFN-γ and IL-17. Zhao *et al*. documented that treatment of cultured endothelial cells with copper-oxidized LDL up-regulated NOX2 expression[64]. Furthermore, Bae *et al*. noted that minimally oxidized low-density lipoprotein, a potent exacerbator of atherosclerosis, facilitates NOX2-dependent generation of ROS in macrophages[65]. Such ROS generation appears to be mediated through the TLR4/PLCγ/PKC axis. Thus, a variety of humoral stimuli, in addition to cytokines, may be responsible for the pro-atherogenic over-activation of NOX systems.

While several studies regarding atherosclerotic diseases employ the context that over-activation of vascular and myeloid NOX facilitate oxidative stress, thereby exacerbating the diseases as noted above, certain finding challenges this hypothesis. Sobey *et al*. recently showed that NOX1 activity is reduced during atherogenesis in western-diet-fed *Apoe*-null mice, and that deficiency of *Nox1* in mice leads to increasing plasma LDL/VLDL and triglyceride levels[66]. Furthermore, *Nox4* deficiency in *Apoe*-null mice results in leukocyte adhesion to ECs and macrophage recruitment to atherosclerotic lesions, thereby accelerating atherogenesis[67]. This may be due to the activation of ECs by depletion of NOX4-derived H_2O_2, which is characterized by the induction of adhesion molecules and inflammatory cytokines in the cells. It was also reported that *Nox4* deficiency did not change the development of atherosclerotic lesions in the streptozotocin-induced diabetic *Apoe*-null mice[60]. Collectively, NOX1 and NOX2 in vascular endothelium and its product $O_2^{\bullet -}$ exert deleterious roles in atherosclerosis, while NOX4 exhibits constitutive and protective effects even in the inflamed vasculature. It is noteworthy that the roles of NOX1 in atherogenesis are complicated, since deficiency of *Nox1* in *Apoe*-null mice

worsens plasma dyslipidemia.

Plaque stability, which is characterized by the thickness of fibrous cap and the degree of inflammation as well as the amount of recruited macrophages in the plaques, is important to predict whether the plaque ruptures. It is well known that unstable plaques often trigger occlusive diseases, including atherothrombosis and acute coronary syndrome. Collagens constitute a major portion of the extracellular matrix in the atherosclerotic plaque and also modulate cellular responses through its specific receptor[68, 69]. Matrix metalloproteinases (MMPs), which are secreted from vascular resident cells and immune cells in the atherosclerotic lesions, are capable of degrading extracellular matrix, including collagen and fibronectin, thereby accelerating the remodeling of extracellular matrix[70]. Deficiency of *Nox1* in *Apoe*-null mice potentiated the secretion of MMP9 and diminished the deposition of collagen and the recruitment of α-SMA-positive cells into the lesions[66]. Similarly, *Nox4* deficiency in *Apoe*-null mice showed reduction of collagen contents in the lesions[67], implying the possible contribution of those NOX family members to plaque stability. However, since the stability is hardly evaluated in the murine atherosclerosis models, the contribution of NOX family members to the plaque stability/instability, in particular *in vivo*, is currently uncertain.

There is no direct evidence regarding atherogenic or atheroprotective actions of NOX5. Nevertheless, cell-based experiments predicted the role of NOX5 in human atherosclerosis. Indeed, NOX5 variants were expressed in human THP-1 monocytes and primary $CD14^+$ monocytes and contributed to ROS generation[71]. NOX5 protein expression, which is inducible by interferon-γ and oxidized LDL, is localized to $CD68^+$ macrophage-enriched human atherosclerotic plaques. Thus, the roles of NOX5 in atherosclerosis are worthy to be evaluated in the future investigations.

SOD catalyzes the conversion of NOX-generated $O_2^{\bullet-}$ to H_2O_2, and reportedly associates with vascular homeostasis[72]. The SOD family is comprised of three isozymes, SOD1, SOD2, and SOD3[73]. SOD1, which is expressed in vascular endothelium, is the major intracellular SOD (cytosolic Cu/ZnSOD), and SOD1 transgenic mice exhibit reduced AngII-induced oxidative stress, MCP-1 induction, and macrophage recruitment in the aorta[74]. Conversely, other research has reported that transduction of SOD1 in high fat diet-fed mice potentiates the formation of atherosclerotic lesions[75]. Thus, the role of SOD1 in atherogenesis may vary depending on animal models. SOD2, which is a manganese (Mn) containing enzyme (MnSOD), is localized in the mitochondrial matrix and catalyzes the dismutation of $O_2^{\bullet-}$ that is generated by the respiratory chain of enzymes[73]. Ballinger *et al.* noted that SOD2 regulates apoptotic signals in ECs and proliferation/apoptotic signals in VSMCs, and that the deficiency of SOD2 in *Apoe*-null mice facilitates the formation of atherosclerosis, which appears to be mediated through the mitochondrial DNA damage[76]. SOD3, a secreted Cu/Zn-containing SOD (ecSOD), is abundant in the extracellular space in the vascular tissue[73] and is mainly derived from macrophages/foam cells as well as VSMCs and fibroblasts in human and mouse atheromas[77, 78]. Sentman *et al.* noted that ablation of SOD3 leads to a slight increase in atherosclerotic lesions in *Apoe*-null mice one month following initiation of an atherogenic diet, while such phenotypes of SOD3-deficient mice vanished after three months on the atherogenic diet or after eight months on standard chow[79]. Thus, it is likely that mitochondrial SOD2 is atheroprotective; however, the roles of SOD1 and SOD3 in atherosclerosis are still unclear.

Aneurysmal Diseases

Oxidative stress induces inflammatory responses even in aneurysmal lesions[80]. This facilitates accumulation of MMPs and subsequent remodeling of vascular tissues, which can be threshold process in some aneurysmal diseases[80]. Indeed, macrophage- and vascular cell-derived MMPs-9 and -12 are considered major exacerbators of aneurysmal diseases[81, 82] because they break down vascular structures, including the elastic lamellae and basement membrane, which leads to vascular dissection in some instances[80]. An early investigation suggested that feeding a diet enriched with antioxidant vitamin E reduces aortic dissection and subsequent aortic expansions in AngII-administrated *Apoe*-null mice[83]. Thus, oxidative stress facilitates pathogenesis of aneurysmal diseases in mice. In human subjects, induction of $p22^{phox}$-based NADPH oxidase and elevation of ROS are evidenced in aneurysmal aortic walls, which is significant in the vascular regions where chymase-positive mast cells and monocytes/macrophages were enriched[84]. MMP activity appears intense in such inflamed lesions[84]. Interestingly, multiple regression analysis showed that the therapeutic interventions using statin or angiotensin II type I receptor blocker (ARB) abrogates $p22^{phox}$ induction[84]. Other researchers showed that plasma malondialdehyde levels are elevated in patients with AAA compared with those in the control group, indicating systemic elevation of oxidative stress in AAA patients[85]. Oxidative stress in human AAA segments is reduced by either the addition of the NOX inhibitor apocynin or diphenyleneiodonium, the cyclooxygenase inhibitor indomethacin, or the nitric oxide synthase inhibitor L-NAME or 1400W[85]. Further-

Table 2. Genotype of NOX and pathogenesis of AAA in mice

Isozyme (gene symbol)	Genotype and diet/treatment	Aneurysmal phenotype	Other phenotype	Ref.
NOX1 (*Nox1*)	*Nox1*$^{-/y}$ AngII infusion	Aortic dissection ↓	Aortic TIMP-1 ↑	89
NOX2 (*Cybb*)	*Cybb*$^{-/y}$*Ldlr*$^{-/-}$ Cholesteol diet (0.15% cholesterol) AngII infusion	Incidence of dissecting aneurysms ↑ Aortic diameter ↑	Conversion to M1 macrophage ↑ IL-1β ↑ MMP-9/12 ↑ Collagen IV contents ↓ ROS in macrophages ↓	91
	CYBB-Tg *Apoe*$^{-/-}$ AngII infusion	Aortic dissection ↑	Aortic ROS ↑ Aortic VCAM-1 ↑ Aortic leukocyte recruitment ↑ Aortic MMP activity ↑ Aortic cyclophilin A ↑	90
p47^{phox} (*Ncf1*)	*Ncf1*$^{-/-}$*Apoe*$^{-/-}$ AngII infusion	Incidence of dissecting aneurysm ↓ Aortic diameter ↓	AngII-induced hypertension ↓ Aortic ROS ↓ Aortic macrophage recruitment ↓ Aortic MMP activity ↓	88

more, NADPH-stimulated production of ROS significantly correlated with diameter of AAA[85]. Expression levels of NOX1, NOX2, and NOX5 are elevated in the AAA segments in addition to p22^{phox}, while that of NOX4 declined; thus, NOX1, NOX2, and NOX5 may be candidate responsive enzymes in human AAA[85].

Gene-targeting studies have disclosed the subtype-specific contribution of NOX to aortic dissection and pathogenesis of aneurysmal diseases (**Table 2**). Contribution of NOX in AAA was mainly investigated by using an AngII-infused hyperlipidemic mouse model, which was established by Daugherty *et al.*[86]. It is noteworthy that aortic dissection frequently precedes aortic expansions in this animal model[87]; thus, the term "dissecting aneurysm" is used in this manuscript instead of "AAA" when it applies to the mouse model. Ablation of *Ncf1* (p47^{phox} gene) leads to the reduction of systolic blood pressure as well as the incidence of aortic expansion in AngII-administrated *Apoe*-null mice[88]. Co-administration of vasopressor phenylephrine together with AngII recovers blood pressure in *Ncf1*-deficient mice, whereas it does not affect the incidence and aortic expansion. This indicates that p47^{phox} potentiates dissecting aneurysms independently of blood pressure. Loss of p47^{phox} suppresses macrophage recruitment into aneurysmal lesions and activates MMP2. As a result, p47^{phox}-mediated ROS is likely to trigger over-activation of MMP2, which decays elastic lamella, thereby reducing vascular stiffness in the lesions. Similar to p47^{phox}, deficiency of *Nox1* reduces incidence of aortic dissection in AngII-infused wild type mice[89]. In this case, up-regulation of tissue inhibitor of metalloproteinase-1 (TIMP-1), an endogenous inhibitor of MMPs, is capable of suppressing MMP activity. Collectively, excess activity of NOX1 and its organizer protein p47^{phox} augment dissecting aneurysms by accelerating MMP-dependent ECM turnover.

Contribution of p47^{phox} to dissecting aneurysms[88] predicts the substantial effects of NOX2, another partner of p47^{phox}, on the disease. Indeed, Fan *et al.* noted that EC-specific transduction of exogenous *CYBB* (NOX2 gene) facilitates aortic dissection in AngII-infused mice[90]. We recently investigated the roles of endogenous NOX2 in dissecting aneurysms using gene-targeting study[91]. However, unexpectedly, systemic and myeloid-specific deficiency of *Cybb* promotes aortic expansion, but reduces the level of ROS in aneurysmal lesions in AngII-infused mice. *Cybb* deficiency stimulates macrophage conversion toward the M1 subset, enhancing expression of IL-1β and MMP-9/12 mRNA in lesions. Administration of a neutralizing antibody against IL-1β abolishes aneurysmal development in *Cybb*-deficient mice; thus, IL-1β may be key mediator for this phenotype. Isolated bone marrow-derived macrophages from *Cybb*-null mice could not generate ROS. Alternatively, IL-1β expression in peritoneal and bone marrow-derived macrophages, but not in peritoneal neutrophils, is substantially enhanced by *Cybb* deficiency, which is largely due to the up-regulation of STAT1 in the cells. Fur-

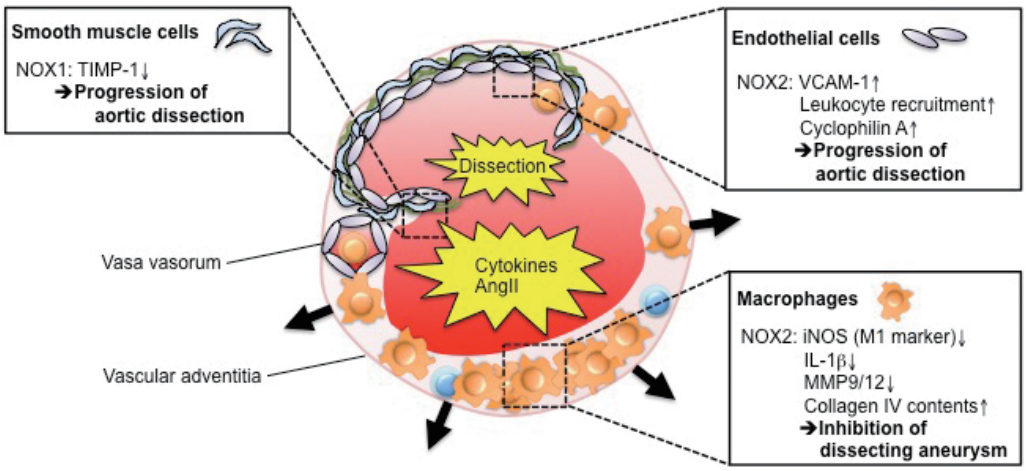

Fig. 3. Roles of vascular NOX systems in aortic dissection and aneurysmal diseases

NOX1 acts as an exacerbator of aortic dissection, owing to its suppressive effects on TIMP-1 in vascular smooth muscle cells. Similarly, over-activation of NOX2 in vascular endothelial cells augments aortic dissection by inducing cyclophilin A and VCAM-1 expression, resulting in subsequent recruitment of leukocytes. By contrast, loss of NOX2 drives adventitial macrophages toward M1 subset, leading to the up-regulation of IL-1β and MMP9/12 in the cells. Accordingly, it is likely that systemic deficiency of NOX2 in mice augments dissecting aneurysms by the compensatory activation of adventitial macrophages. MMP: matrix metalloproteinase.

thermore, MMP9 secretion in bone marrow-derived macrophages is constitutively elevated, owing to the up-regulation of NF-κB signals. Thus, it appears that *Cybb* deficiency enhances multiple inflammatory cascades. Since MMP9 plays a leading role in maintaining ECM turnover in aneurysmal lesions[81], up-regulation of MMP9 by *Cybb* deficiency may participate in the pathogenesis of dissecting aneurysms. Compensatory activation of phagocyte inflammatory systems by the loss of NOX2 is not surprisingly, since over-activation of phagocytes by ablation of NOX systems has been reported in the literature[1, 92-94]. Indeed, genetic defects in phagocytic NOX systems have been found to induce chronic granulomatous disease (CGD), with most CGD patients experiencing systemic sterile hyperinflammation[1]. A deficiency in *Cybb* or *Ncf1* in mice was found to significantly up-regulate LPS-induced IL-1β expression in lung, accompanied by the activation of AP-1 and NF-κB transcriptional systems[92]. Moreover, a loss-of-function mutation in $p47^{phox}$ causes inflammatory skin lesions in mice concomitant with the up-regulation of IL-1β, which are suppressed by the genetic reintroduction of wild-type $p47^{phox}$[93]. Monocytes from CGD patients lacking *Ncf1* have been found to produce higher amounts of IL-1β in response to LPS than monocytes from healthy subjects, an increase likely owed to the up-regulation of caspase-1-based inflammasomes[94]. Thus, deficiency of phagocyte NOX systems is compensated by the alternative inflammatory signals including NF-κB, AP-1, and JAK/STAT signals under certain conditions. IL-1β can be accounted as a key element for such compensation. Reasons for the compensatory activation of other inflammatory signals in NOX-deficient phagocytes are still unclear; however, considering that phagocytes play central roles in innate immunity, it is not surprisingly that their host defense machinery is redundant. We suspect that such alternative host defense mechanisms, particularly in macrophages, are interrelated to the pathogenesis of dissecting aneurysms. Such compensatory action is not observed in resident vascular cells[91]; thus, the redundancy may be unique in phagocytes. It is noteworthy that NOX2 is expressed also in vascular endothelium in atherosclerotic lesions, and the lack of endothelial NOX2 improves bioavailability of $NO^{\bullet}$, thereby suppressing the formation of lesions[61]. Although rising bioavailability of $NO^{\bullet}$ similarly ameliorates dissecting aneurysms in mice[95], *Cybb* deficiency does not reduce dissecting aneurysms. Thus, macrophage over-activation, rather than recovery of $NO^{\bullet}$ bioavailability in the endothelium, may be predominant in the aneurysmal diseases in *Cybb*-deficient mice.

Cell-type-specific roles of NOX isozymes in dissecting aneurysms are summarized in **Fig. 3**. Based on the gene-targeting data in mice, NOX2 in ECs[90] and NOX1 in VSMCs[89] may contribute to aortic dissection in the aorta. Whereas EC-specific transduction of *Cybb* is capable of inducing aortic ROS and adhesion

molecules in murine aneurysmal lesions, thereby accelerating aortic dissection[90], deficiency of endogenous *Cybb* does not reduce dissecting aneurysms[91]. Thus, it is thought that macrophage over-activation, rather than reduction of EC inflammation, may be significant in the formation of dissecting aneurysms in *Cybb*-deficient mice. In contrast, *Nox1* deficiency results in the elevation of TIMP-1 expression and the reduction of aortic dissection. TIMP-1 is secreted by VSMCs; thus, NOX1 in VSMCs potentially augments aortic dissection.

Our data shows that over-activation of macrophages by *Cybb* deficiency accelerates pathogenesis of dissecting aneurysms[91], although the deficiency of *Ncf1* reportedly ameliorates the diseases[88]. Whereas the reasons for the opposite aneurysmal phenotypes between *Cybb*- and *Ncf1*-deficient mice are currently unknown[88, 91], we speculate that the suppression of NOX1, another partner of $p47^{phox}$, contributes to the ameliorative phenotypes of *Ncf1*-deficient mice. The compensatory over-activation of macrophages by *Nox1* deficiency was not reported so far[89], while deficiency of *Ncf1* as well as *Cybb* reportedly leads to the over-activation of the inflammatory cascades in macrophages. It was reported that NOX1 is abundant in VSMCs but not in macrophages[1] and is largely responsible for the production of superoxide in VSMCs[96]. Consistently, *Nox1* deficiency influences the VSMC functions during AngⅡ-induced aortic dissection in mice[89]. In contrast, NOX2 activity appears to be predominant in phagocytes rather than VSMCs[96]. Accordingly, *Cybb* deficiency mainly influences macrophage functions in the murine dissecting aneurysms[91]. Thus, it is likely that *Ncf1* deficiency leads to the phenotypic changes in both VSMCs and macrophages, which are due to the dysfunction of NOX1 and NOX2, respectively. Considering the amelioration of dissecting aneurysms in *Ncf1*-deficient mice[88], it is speculated that the harmful redox action of NOX1 in VSMCs, rather than the anti-compensatory action of NOX2 in macrophages, is dominant in aneurysmal lesions. While NOX1 and NOX2 are expressed in vascular endotheium[1], the contribution of those NOX isozymes to EC functions during dissecting aneurysm is still enigmatic[89, 91].

Conclusive Remarks

A recent meta-analysis showed that antioxidants, such as vitamin E and β-carotene, failed to prevent the development of atherosclerosis and related cardiovascular events[97], suggesting that antioxidants may be clinically ineffective in patients with atherosclerotic diseases. Considering the functional diversity of redox-related molecules, clinical approaches targeting specific molecules may be more promising. Based on animal experiments, it is possible that targeting NOX family molecules, except for NOX2 and NOX4, is beneficial as a therapy for atherosclerotic and aneurysmal diseases. Nevertheless, the adverse pro-inflammatory effects of non-subtype-selective NOX inhibitors should be carefully monitored when they serve as candidate drugs in the clinical trial.

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Conflict of Interest

None.

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