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Microvascular NADPH oxidase in health and disease

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Abstract

The systemic and cerebral microcirculation contribute critically to regulation of local and global blood flow and perfusion pressure. Microvascular dysfunction, commonly seen in numerous cardiovascular pathologies, is associated with alterations in the oxidative environment including potentiated production of reactive oxygen species (ROS) and subsequent activation of redox signaling pathways. NADPH oxidases (Noxs) are a primary source of ROS in the vascular system and play a central role in cardiovascular health and disease. In this review, we focus on the roles of Noxs in ROS generation in resistance arterioles and capillaries, and summarize their contributions to microvascular physiology and pathophysiology in both systemic and cerebral microcirculation. In light of the accumulating evidence that Noxs are pivotal players in vascular dysfunction of resistance arterioles, selectively targeting Nox isozymes could emerge as a novel and effective therapeutic strategy for preventing and treating microvascular diseases.

Keywords

Microcirculation; NADPH oxidase; Reactive oxygen species; Endothelial dysfunction; Arterioles; Remodeling; Myogenic tone; Aging

1. Introduction

1.1. Microcirculation

The microcirculation, comprised of the most distal segments of the vascular system, proceeds from an active network of arterioles to capillaries and venules in the periphery. Together, these vascular beds function to regulate blood flow and perfusion pressure. The microcirculation is responsible for optimizing the supply of nutrients and oxygen to various organs, while simultaneously removing metabolic waste products. Consequently, the rate of blood flow through these beds is tightly controlled by the metabolic demands of the tissue they pervade and is finely tuned by the more proximal small arteries and resistance arterioles. It is widely acknowledged that the small arteries and arterioles account for the majority (80%) of vascular resistance to blood flow [1–3]. They also serve to protect downstream capillaries from the potential damaging effects of high perfusion pressure and,

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Conflict of interest

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in turn, prevent end-organ damage [4]. Arteriolar lumen size can vary dramatically depending on various species, distinct vascular beds and intrinsic contractile status. However, the common feature shared by arterioles across all vascular beds and species is the fact that they only possess one or two layers of smooth muscle cells in their arterial wall, which play a remarkably effective and fundamental role in regulating vascular contractility and local perfusion. Regulation of arteriolar diameter and blood flow in the microcirculation is a dynamic and well-controlled process that integrates signals from mechanical, chemical, hormonal and neuronal stimuli. This is also achieved by an ensemble of responses elicited by endothelial cells and smooth muscle cells [1,2]. Impaired arteriolar function, as observed in various cardiovascular pathologies such as hypertension, diabetes, stroke and aging, leads to vascular remodeling, tissue ischemia and organ damage [3,5–8]. Emerging evidence has shown that changes in the oxidative environment are often accompanied by the aforementioned vascular dysfunction and cardiovascular diseases in the microcirculation [6,9–12]. As a primary source for oxidative stress and reactive oxygen species (ROS) in vascular cells, the contribution of NADPH oxidases (Noxs) to the cardiovascular system in health and disease has been investigated in a plethora of studies [9–11,13–15]. In the microcirculation, Noxs are implicated in a variety of vascular pathologies including arteriolar remodeling [16,17] and endothelial dysfunction [18,19] in systemic microvessels, as well as impaired neurovascular coupling [20] and disrupted blood-brain barrier (BBB) integrity [21] in the cerebral circulation. The current review aims not to be an exhaustive review on the roles of all sources of oxidases in the microcirculation. Rather, the goal here is to survey what is known of the roles of Noxs in ROS generation in resistance arterioles and to summarize their contributions to microvascular physiology and pathophysiology in both cerebral and systemic microcirculation.

1.2. NADPH oxidases (Noxs) in the microvasculature

The nicotinamide-adenine-dinucleotide phosphate (NADPH) oxidases (Noxs) are an increasingly complex family of proteins whose core components are membrane bound. All Noxs transfer electrons from NADPH to molecular oxygen, thus generating superoxide anion and attendant other downstream ROS with NADP^+ as a byproduct [22]. There are seven members of the Nox family characteristically distinguished and referred to by their catalytic core component, namely prototypic Nox2 (previously gp91^{phox}), Nox1, Nox3, Nox4, Nox5, Duox1 and Duox2. These isozymes are heterogeneously distributed across tissue. Their structure and function are complex and have been extensively reviewed elsewhere [9,10,12–14,23]. The focus here will be on their profound implications in vascular function and disease in the microcirculatory system.

Of the seven members of the Nox family, Nox1, Nox2, Nox4 and Nox5 are known to be expressed in human vascular tissue. They are considered the “professional” enzymatic source of ROS in vascular cells, and are found to exert varied and often distinct physiological functions depending on the cell, tissue and/or species (Table 1). For instance, Noxs identified by their core catalytic subunits, i.e. Nox2 and Nox4, are involved in proliferation of endothelial cells [24], whereas Nox1 facilitates smooth muscle cell mitogenesis and proliferation and thus likely aids in cell replenishment under normal conditions [25]. Smooth muscle Nox4 has been implicated widely in the maintenance of a

differentiated phenotype [26,27]. Further, Noxs are also involved in regulation of vascular contractility [28–30]. On the other hand, Nox isozymes are also known to be major players in mediating vascular dysfunction and disease [9,11–13,23,31–33].

Membrane-integrated Nox subunit p22^{phox} is required by Nox1, Nox2 and Nox4 but not the Nox5 isoform for membrane stabilization and activity [23]. The prototypical phagocytic NADPH oxidase is composed of five subunits: p47^{phox} (organizer), p67^{phox} (activator), p40^{phox}, p22^{phox} and catalytic subunit Nox2 (previously gp91^{phox}) [34–37]. p47^{phox} is phosphorylated upon stimulation, and hence the cytosolic units form a complex that includes the small Rho-family GTP-binding protein Rac1/2 [38]. This complex is then well characterized as being translocated to the plasma membrane where it interacts with the heterodimeric flavoprotein (cytochrome b558 complex) formed by p22^{phox} and Nox2. Once assembled, the enzyme is rendered active and generates superoxide anion from molecular oxygen [23]. The Nox2 oxidase complex is not only widely distributed in phagocytes but is ubiquitous in lung, heart and vasculature [23]. In the vasculature, it was first discovered and characterized in fibroblasts [28,36,39,40], vascular smooth muscle cells [41–43], and endothelial cells [44,45]. These findings sparked exploration and discovery of Nox in virtually all cell types and led to the knowledge that similar orthologues are highly conserved in nature dating to some of their earliest evolutionary predecessors in fungi; and resembling the subunit-modulated Noxs that are highly regulated in mammals [46]. Being the first to be discovered, Nox2 remains to be the most extensively studied member of the Nox family in numerous physiological and pathological processes. In the microvasculature, Nox2 appears to be mostly present in the endothelium and adventitia [20,24], responsible for endothelial dysfunction under disease conditions [19,47] but has been shown by Touyz et al. to also be functionally active in human resistance smooth muscle cells [43]. Similar to Nox2, the canonical Nox1 requires binding of p22^{phox}, p47^{phox} homologue NoxO1, p67^{phox} homologue NoxA1 and Rac1/2 for activation [48]. Nox1 oxidase complex is most abundant in colon, prostate, uterus and vascular cells [23]. In contrast to Nox1 and Nox2 complexes, activity of Nox4 was shown to be modulated by the enzyme polymerase delta-interacting protein or Poldip2 [49]. Also distinguishing itself from other Nox complexes, Nox4 appears to be constitutively active and to most favorably produce hydrogen peroxide over superoxide anion [23]. Interestingly, Nox4 is substantially higher in the cerebral circulation and purportedly generates hydrogen peroxide that modulates arteriolar activity as a potent vasodilator under physiological conditions [50,51]. However, the findings in that study using a selective Nox2 inhibitor along with apocynin might challenge the notion of a singular role for Nox4 and perhaps suggest a cross-talk between Nox2 and Nox4 in this vasodilator effect [52]. Nox5 activity does not depend on p22^{phox}, nor cytosolic organizer or activator. Rather, it is regulated by calcium binding through EF-hand motifs [53]. As the most recently discovered Nox member, the expression and functional significance of Nox5 are poorly appreciated since Nox5 is absent in mouse and rat genomes [54]. In the microcirculation, Nox5 isozyme is found in porcine and human coronary resistance arterioles [55–57]. Additionally, its expression is significantly enhanced in these microvessels under disease conditions such as atherosclerosis and myocardial infarction, suggesting that it may be an important mediator of the pathological degeneration of human resistance arteries [56,57].

In this review, we discuss the involvement of Noxs in two distinct microvascular systems: systemic microcirculation and cerebral microcirculation. In each section, we will briefly summarize the expression of Noxs in various vascular beds and cell types. Further, implication of Noxs in both physiological processes such as myogenic tone regulation and vasodilator mechanisms, and cardiovascular pathologies including endothelial dysfunction and vascular remodeling will be discussed in detail. In light of the burgeoning evidence that Noxs are central players in vascular dysfunction of resistance arterioles, targeting Nox could emerge as a novel and effective therapeutic strategy for preventing and treating microvascular diseases.

2. Systemic microcirculation

2.1. Nox expression

In systemic microvessels, expression of Nox isozymes has been described in a number of resistance vascular beds, participating in physiological and pathological processes. For example, mRNA transcript and protein of Nox1, Nox2, Nox4, p22^{phox}, p47^{phox}, p67^{phox} and Rac1/2 subunits were detected in mouse and rat mesenteric resistance arteries [19,58,60,66,67,71], where Noxs are implicated in structural and functional alterations in response to distinct stimuli [17,72,73]. In mouse coronary arteries, protein expression of Nox2 and its regulatory subunits p67^{phox}, p47^{phox} and p22^{phox} were reported [64]. Moreover, immunohistochemical assays illustrate that Nox1, Nox2, Nox4, p22^{phox}, p47^{phox} and p67^{phox} are expressed in human coronary arteriolar endothelium [62]. In addition, Nox5, both at the level of mRNA transcript and protein, was detected in human coronary arterioles. Immunofluorescence staining of Nox5 corroborated its localization in both endothelium and smooth muscle with low expression levels in healthy subjects and high levels in patients with coronary artery disease and myocardial infarction [56,57]. Recently, Nox5 mRNA was also detected in porcine coronary artery smooth muscle cells, mediating growth factor-induced expression and activation of intermediate conductance Ca²⁺-activated K⁺ channels (KCNN4) and subsequent smooth muscle cell migration [55]. Noxs are also involved in the microvascular function in the kidney, where Nox2 was found in mouse afferent arteriole, contributing to angiotensin II (AngII)-induced arteriolar contraction [65]. Furthermore, Nox2 and Nox4 transcripts are found in human renal arterioles, and they modulate endothelium-dependent dilation in elderly patients [68]. Other resistance arteries that express functional Nox isozymes or its regulatory subunits include human subcutaneous arterioles from gluteal and abdominal regions [43,74]. Although the molecular evidence for Noxs in cremaster arterioles is lacking, they appear to be present and functionally active in this vascular bed since ROS scavenger Tempol and Nox inhibitor apocynin inhibited ROS production and downstream inward remodeling of rat cremaster arterioles [75]. Overall, accumulating evidence suggest that Nox activity is crucial for modulations in vascular structure and function in the systemic microcirculation.

2.2. Systemic microvascular Nox in health

The myogenic response is one of the key means by which resistance arteries autoregulate. This crucial hallmark characteristic of arterioles protects downstream capillaries from potential detrimental effects of high intraluminal pressure, thus preventing end-organ

damage due to pressure and perfusion fluctuations. Autoregulation is defined as a process by which resistance arteries constrict and reduce their diameter in response to increased intravascular pressure [76]. Mechanisms of the development and maintenance of pressure-induced vasoconstriction have been rigorously studied for decades. Elevation in intraluminal pressure leads to stimulation of depolarizing influence such as transient receptor potential (TRP) channel opening [77,78], and simultaneous inhibition of hyperpolarizing factors including different types of potassium channels in the smooth muscle cells [79,80]. This results in smooth muscle cell membrane depolarization, Ca^{2+} influx and smooth muscle contraction [76]. Though several aspects of the mechanism underlying pressure-induced vasoconstriction remain to be elucidated, emerging evidence has pointed to the possible contribution of Nox and ROS in the microvasculature under both physiological and pathological conditions. For example, a study showed that elevated transmural pressure initiated a rapid and transient ROS production in isolated resistance arteries from hamster gracilis muscle [29]. Further, pressure-elicited ROS, enhanced Ca^{2+} sensitivity and vascular contraction were all attenuated in the presence of diphenylene iodonium (DPI) or Nox2-selective peptide inhibitor Nox2ds-tat (aka gp91ds-tat) demonstrating that Nox2-derived ROS is physiologically critical for enhancing Ca^{2+} sensitivity and smooth muscle contractility in response to increased intraluminal pressure [29]. Using renal afferent arterioles, another study demonstrated that pathologically enhanced myogenic vasoconstriction is attributed to a 4-fold increase in ROS production in vessels from the spontaneous hypertensive rats compared to control animals [81]. The source of ROS is believed to be Nox2 isozyme, as the superoxide scavenger Tempol and the Nox2 inhibitor gp91ds-tat attenuated pressure-induced vasoconstriction in resistance arterioles [81]. Further, the mechanosensitivity of Nox is strongly suggested as several groups reported that superoxide production is largely stimulated by mechanical stretch in cultured vascular smooth muscle cells [82–84]. Importantly, Nox appears to be the predominant source of ROS in these settings, since Nox inhibitors rather than xanthine oxidase or cyclooxygenase inhibitors effectively attenuated stretch-induced ROS generation [29,82,84]. In agreement with these findings, deleting p47^{phox} as well as reducing Nox1 levels abrogated the response in vascular smooth muscle cells, indicative of a clear role of Nox in stretch-mediated ROS production as well as downstream signaling events [83].

Mechanistically, mechano-activation of Nox is mediated by protein kinase C (PKC), which phosphorylates p47^{phox} and promotes its translocation and interaction with Nox2 [85]. In addition, ROS is found to activate downstream RhoA/Rho kinase signaling in vascular smooth muscle [86,87]. Interestingly, both PKC and Rho kinase pathways are well-established cellular mechanisms that mediate myogenic tone development both at the level of promoting smooth muscle depolarization/ Ca^{2+} entry and enhancing Ca^{2+} sensitivity [88–91], suggesting possible crosstalk involving Nox2 and the protein kinases to facilitate myogenic responses in vascular smooth muscle cells. Moreover, emerging evidence has pointed to the mechano-sensitivity of G_q protein-coupled receptors such as Ang II receptors and P2Y purinergic receptors in resistance arteries [92,93]. It was suggested that osmotically induced cell membrane stretch initiates a conformational modification in Ang II receptors where transmembrane segment 7 enters the ligand-binding pocket to cause receptor activation [94]. Mechano-activation of Ang II receptors as well as other G_q protein-coupled

receptors further stimulate downstream PKC or Rho kinase pathways [91–93,95,96]. Because AngII receptor activation is also strongly associated with Nox activation and ROS production in smooth muscle cells, it is conceivable that complex cellular interactions among AngII receptors, Nox and protein kinases may be essential for developing and maintaining microvascular myogenic tone physiologically, and adversely regulating the contractile responsiveness under disease conditions. Furthermore, since Nox5 possesses EF-hands and is exquisitely sensitive to rises in Ca^{2+} [53], PKC and Rho kinase-mediated increase in Ca^{2+} influx and Ca^{2+} sensitivity may be particularly relevant. In fact, it was demonstrated that PKC phosphorylates Nox5 at key residues, and facilitates enzyme activation as well as ROS production by increasing its sensitivity to Ca^{2+} [97,98]. However, the physiological significance of this pathway, particularly its participation in vascular function requires further investigation.

2.3. Systemic microvascular Nox in disease

2.3.1. Microvascular remodeling—In addition to rapid contractile and dilatory responses to mechanical or chemical stimuli, resistance vessels also display adaptive structural modifications that alter their diameter and responses to vasoactive molecules [99]. Vascular remodeling is controlled by a number of processes including cytoskeletal remodeling, cell growth, cell proliferation, cell migration, apoptosis, and extracellular matrix modification, all of which are influenced by redox state [2]. The involvement of ROS in different mechanisms of the vascular remodeling process is more thoroughly discussed elsewhere [2]. This section of the review will focus on Nox-mediated inward and outward remodeling at the level of microcirculation. Vascular remodeling can be induced by various factors, such as mechanical and hemodynamic forces, and neurohumoral or paracrine agents. Inward hypertrophic remodeling is believed to commonly occur in resistance arteries that undergo prolonged contraction as reported in cardiovascular diseases such as hypertension and diabetes [100,101]. It involves cell proliferation and alterations in the amount and composition of extracellular matrix components which eventually reduce lumen diameter and increase vascular wall thickness, media/lumen ratio and cross-sectional area [100]. In contrast to inward remodeling that is caused by prolonged contraction as a primary stimulus, outward remodeling happens when vessels are subjected to chronically increased blood flow or shear stress, and they adaptively outward remodel to meet the need for higher metabolic demand [102]. These arterioles exhibit increased wall thickness and vascular diameter as well as an increased luminal size. Both inward and outward hypertrophy are important compensatory mechanisms as they occur when vascular myogenic reactivity is not sufficient to normalize circumferential stress [2]. Also similar to myogenic autoregulation, inward hypertrophy serves to protect downstream capillaries from perfusion pressure fluctuations and prevent end-organ damage, whereas outward remodeling functions to meet higher metabolic demand that is more commonly seen during pregnancy and exercise training [103,104]. Multiple studies have implicated Nox in both types of remodeling in the microvasculature. For example, it was shown that inward hypertrophy of cremaster and mesenteric arterioles induced by prolonged exposure to vasoactive compounds, primarily AngII, is accompanied by increased superoxide anion production [16,17,72,75,105]. Both ROS generation and vascular remodeling could be prevented by the non-specific Nox inhibitor apocynin, suggestive of an important role of Nox in the microvascular remodeling

mechanisms [16,17]. In accordance with this notion, Briones et al. inferred that treatment with atorvastatin attenuates AngII-elicited mesenteric resistance artery hypertrophy partially by inhibiting AngII-induced increase in Nox1 expression [17]. Additionally, involvement of Nox in high flow-induced outward remodeling has been evaluated. That is, high flow-mediated ROS production and diameter enlargement of mesenteric resistance arteries were inhibited by Tempol and apocynin [106]. Potentiated p67^{phox} and Nox2 expression levels in these arterioles further suggested that Nox-derived ROS are responsible for flow-initiated outward remodeling in the microcirculation [106]. Similarly, Castier et al. found that prolonged stimulation by high blood flow facilitated artery enlargement and concomitant shear stress reduction from the initial surge [107]. Surprisingly, deleting Nox2 had little effect to reverse these alterations, whereas p47^{phox} deletion conferred significant improvement in artery caliber and shear stress, and elicited pronounced decreases in oxidative stress. Those findings suggested that outward remodeling is mediated by a p47^{phox}-dependent signaling pathway which is independent of Nox2 activation [107]. This could suggest that the hybrid Nox1 system that utilizes p47^{phox} is operant here. In both the inward and outward remodeling processes, it is generally accepted that ROS participates in part via activating matrix metalloproteinases (MMPs), including MMP2 and MMP9 [75,107,108]. Castier et al. proposed that increased superoxide anion and NO and, in turn, peroxynitrite subsequently activate these MMPs and cause vascular remodeling in the microvasculature [107]. However, the specific Nox isozymes or the downstream signaling cascades that facilitate these types of hypertrophy have not yet been identified.

2.3.2. Endothelial dysfunction—Endothelial dysfunction is characterized by a shift in vascular function towards reduced vasodilation, a pro-inflammatory state, and pro-thrombotic properties. This shift is implicated in many types of cardiovascular diseases, such as hypertension, coronary artery disease, chronic heart failure, peripheral vascular disease, diabetes and chronic kidney failure [109]. At the level of microcirculation, endothelial dysfunction is believed to play a key role in the development of atherosclerosis, abnormal angiogenesis, vascular leakage and stroke, all of which are associated with enhanced oxidative stress [110]. Noxs, a primary cause of oxidative stress in the vasculature, have major implications for endothelial dysfunction in the microcirculation by way of producing ROS and subsequently disrupting NO signaling. The diffusion-limited reaction between superoxide anion and NO reduces NO bioavailability and diminishes its anti-proliferative, anti-coagulant, anti-inflammatory and vasodilatory properties. The product of this reaction, peroxynitrite, is in and of itself a powerful oxidant and can exacerbate vascular dysfunction by causing further damage to lipids, proteins and DNA, uncoupling endothelial nitric oxide synthase (eNOS) and diminishing smooth muscle responses to NO [111,112]. In the systemic microvasculature, impaired endothelium-dependent vasodilation and concomitant enhanced Nox-mediated oxidative stress have been observed in microvascular coronary arterioles and mesenteric resistance arteries in a number of cardiovascular diseases, such as obesity [113,114], diabetes [18,19,47,115,116], hypertension [117–119] and ischemia-reperfusion [73]. In these studies, elevated expression of Nox subunits is a common characteristic across different cardiovascular pathologies. For example, ischemia-reperfusion injury induced upregulation of Nox1 and p47^{phox} mRNA transcript levels, which purportedly accounts for superoxide production and potentiation of endothelin 1-induced

vasoconstriction [73]. In a mouse model of type 1 diabetes, isolated mesenteric resistance arteries showed a 4-fold increase in Nox activity, and a 4–6 fold enhancement of Nox2 and Nox4 expression compared to vessels from control mice. Moreover, those findings are consistent with Nox being functionally involved in a largely attenuated endothelium-dependent vasodilation [120]. Similarly, mesenteric arterioles obtained from young type 2 diabetic rats showed higher ROS and greater protein expression of Nox2 isozyme compared to young lean rats. ROS production is even more pronounced in aged diabetic rats, in which elevation in p67^{phox} subunit expression was also noted [47]. In other type 2 diabetic mouse models, upregulation of other Nox2 subunits, namely p22^{phox}, p40^{phox} and p47^{phox}, is observed in mesenteric and coronary resistance arteries [18,19,115]. Further corroborating a role of Nox2 in endothelial dysfunction, gp91ds-tat (aka Nox2ds-tat)-treated arterioles exhibited improved vasodilation to acetylcholine compared to untreated vessels. Moreover, evidence that knocking out p47^{phox} prevented disruption of endothelium-dependent vasodilation is consistent with the essential contribution of p47^{phox} to microvascular dysfunction [19]. Several mechanisms underpinning Nox activation and subsequent endothelial dysfunction have been proposed. In diabetic and hypertensive disease models and obese patient samples, TNF α and endothelin-1 were upregulated [114,115,121,122], leading to amplified Nox-mediated oxidative stress and microvascular dysfunction. In a type 2 diabetic mouse model, superoxide anion levels are augmented by the interaction between advanced glycation end products (AGE) and receptor for AGE (RAGE), both of which are heavily involved in pathophysiology of hyperglycemia and diabetes. AGE-RAGE binding further potentiated expression of TNF α , oxidative stress and endothelial dysfunction in coronary microvessels [115]. In another type 2 diabetes study, augmented epidermal growth factor receptor (EGFR) tyrosine kinase was shown to enhance Nox expression and activity, which facilitated an increased vasoconstrictor response and attenuated acetylcholine-induced dilatation [19]. Although the mechanistic implications of ROS signaling pathway in microvascular endothelial dysfunction is not fully understood, it is clear that Nox, particularly Nox2, plays a central role in this pathological process. Therefore, selectively targeting Nox or its stabilizing membrane counterpart p22^{phox} can be a useful strategy in terms of restoring the balance between pro- and anti-oxidants and improving vascular functionality. In fact, one study showed that p22^{phox} small interfering RNA (siRNA) significantly improved endothelium-dependent vasodilator responses in microvessels isolated from diabetic mice [18]. This appears to be mediated by reversal of the augmented phosphorylation of downstream mitogen-activated protein kinases (MAPKs) p38 and extracellular signal-receptor kinase (ERK) following p22^{phox} knockdown [18]. Since it is well established that activation of MAPKs is a fundamental mechanism responsible for cardiovascular dysfunction in diabetes, p22^{phox} siRNA-mediated decrease in phosphorylation of these kinases is expected to effectively ameliorate vascular abnormalities [123,124]. Taken together, the findings support the notion that Nox is a central player and a potential drug target for treating microvascular diseases.

2.3.3. Microvascular angiogenesis—Angiogenesis is a natural process in the microcirculatory system. It is induced by ischemia and is characterized by the sprouting of new blood vessels from existing vessels, as well as the proliferation and migration of microvascular endothelial cells [125]. Several studies have reported that Noxs participate

importantly in the regulation of angiogenesis of the microvascular endothelial cells. In particular, Nox4 was found to be a key mediator of pro-angiogenic signaling through distinct cellular pathways [126–128]. For example, blood flow recovery after hind limb ischemia was significantly enhanced in mice overexpressing human Nox4 and was impaired in mice overexpressing a dominant negative form in the endothelium, suggesting that Nox4 is involved in angiogenic response *in vivo* [129]. In isolated cardiac microvascular endothelial cells, Nox4 expression and activity were substantially increased after cells were subjected to hypoxia/reoxygenation [127]. Transcriptionally downregulating Nox4 exacerbated hypoxia/reoxygenation-induced cell apoptosis and inhibited the expression of hypoxia-inducible factor (HIF-1 α) and vascular endothelial growth factor (VEGF). This suggests that Nox4 promotes microvascular endothelial cell survival, migration and angiogenesis after ischemia/reperfusion injury by activating the HIF-1 α /VEGF signaling [127]. Other mechanisms underlying Nox4-promoted angiogenic response include activation of receptor tyrosine kinases and the ERK pathway [128], and stimulation of transforming growth factor β 1 (TGF β 1) to initiate activation of VEGF receptor 2, p38 MAPK and adenosine monophosphate-activated protein kinase alpha (AMPK α) in microvascular endothelial cells [129]. In addition, there is evidence that Nox2 is an important regulator of angiogenesis *in vivo* [130]. In particular, Nox2 knockout mice exhibited significantly impaired neovascularization in the ischemic hind limb compared to wild type mice. New blood vessel formation in response to ischemia is an important adaptive response for preserving tissue integrity [131]. Therefore, both Nox4 and Nox2 appear to be protective by orchestrating complex angiogenic signaling pathways in endothelial cells of the microcirculation. However, an exaggerated angiogenic response can be pathological, and be associated with tumor growth and metastasis. A large body of evidence appears to suggest that Nox-derived ROS may function as signaling molecules that mediate growth-related responses and facilitate tumor angiogenesis [132] although much more work appears necessary to test this directly.

2.3.4. Aging—The cumulative process of diverse deleterious changes in the cells and tissues with advancing age progressively impairs function and eventually causes death [133]. Aging is a major risk factor for cardiovascular diseases [133]. Among multiple other instigators of the aging process, ROS and oxidative stress are often described as a common denominator in aging and age-related cardiovascular diseases [9,11,134–136]. As the “professional” enzymatic source for ROS generation in vascular cells, Nox isozymes appear to play a crucial role in age-associated cardiovascular dysfunction including coronary artery disease, atherosclerosis, hypertension and diabetes via activation of redox signaling pathways [70,137–143]. Multiple studies have identified age-mediated alterations in the activity and/or expression levels of Nox subunits and the pivotal contribution of Noxs to impairment of endothelium-dependent vasodilation, i.e. endothelial dysfunction. For example, aortas from aged hypertensive rats exhibited increased ROS formation compared to age-matched control rats, which was sensitive to broad spectrum Nox inhibitors VAS2870, apocynin and DPI. This was accompanied by enhanced protein levels of Nox1 and Nox2, but not Nox4, in aged aortas. Further, acetylcholine-induced vasodilation was largely attenuated in these aortas compared to control, which was improved by VAS2870 and apocynin [144]. Another study comparing aortas from young and middle aged spontaneously hypertensive

rats (SHR) and control Wistar rats showed significantly higher mRNA expression of p22^{phox} and Nox activity in the aortas from older SHR rats, concomitant with lower acetylcholine dilatory responses [145]. Those findings seem to suggest a link between enhanced expression and activity of Noxs and conduit artery endothelial dysfunction associated with aging and hypertension. In the microcirculation, attenuated effects from endothelium-dependent dilatory stimuli were also reported. In aged coronary arterioles, flow and acetylcholine-initiated dilation were significantly diminished [146]. Even though endothelial dysfunction paralleled higher Nox-derived superoxide anion generation in aged coronary microvessels, it appears to be independent of a change in expression of Nox subunits such as p47^{phox}, p67^{phox}, Nox1 and p22^{phox} [146]; statistical power could have been an issue for an apparent but nonsignificant rise in p67^{phox}. Nevertheless, the role of Nox-derived ROS in many other manifestations of microvascular dysfunction associated with the aging process is largely unknown. In fact, the microcirculation undergoes significant functional and anatomical changes with age. Apart from declines in endothelium-dependent vasodilation, for instance, age is also associated with Rho kinase signaling-mediated cutaneous vasoconstriction [147]. Since Rho kinase activation has been shown to be regulated by ROS in vascular smooth muscle cells [86], it seems reasonable to propose that age-induced microvascular constriction is also regulated by Nox-mediated ROS signaling. Furthermore, aging is associated with arteriolar stiffness, decreased vascular density and impaired microvascular organization [5]. Nevertheless, signaling mechanisms that underlie the co-existence of Nox-mediated oxidative stress, aging and microvascular pathologies warrant further investigation.

3. Cerebral microcirculation

3.1. Nox expression

In the cerebral vasculature, expression of NADPH oxidases (Nox1, Nox2 and Nox4) at the level of both mRNA and protein has been confirmed by various studies. Specifically, Nox1, Nox2 and Nox4 were detected in human brain vascular smooth muscle cells [63]. In basilar arterial endothelial cells, Nox1 and Nox4 mRNA are highly expressed, whereas Nox2 transcript is expressed to a far lesser extent [61]. The cytosolic components of Nox including p47^{phox}, p67^{phox}, NOXO1 and NOXA1 were also found in these endothelial cells [61]. Furthermore, mRNA of NADPH oxidase subunits Nox1, Nox2, Nox4, p22^{phox}, p47^{phox} and p67^{phox} were also identified in the cerebral neocortical microvessels including cerebral arterioles and capillaries [59]. Using electron microscopy, Nox2 immunoreactivity was detected in the intima and adventitia of cerebral arterioles [20], which may reflect important involvement of ROS-mediated signaling in regulation of cerebral microcirculation.

3.2. Cerebral microvascular Nox in health

Interestingly, cerebral arteries isolated from various healthy animal models presented markedly higher NADPH oxidase activity and ROS generation compared to systemic vessels [50,148], which may be explained at least in part by elevated expression of Nox2 and Nox4 in the cerebral vasculature [50]. It is tempting to speculate that perhaps more significant roles of Nox and ROS are operant in modulating cerebrovascular function and local blood flow under physiological conditions. Indeed, compelling evidence exists for ROS,

particularly hydrogen peroxide (H_2O_2), participating in control of arteriolar reactivity as a potent endogenous vasodilator in the cerebral circulation [50,51,149]. This is supported by the observation that NADPH-induced vasodilation can be abolished by the H_2O_2 scavenger catalase and Nox inhibitor apocynin, indicating that H_2O_2 , generated secondarily from Nox2 and superoxide anion dismutation, is a potent vasorelaxant. As several endogenous vasoactive stimulants such as AngII and TNF α elicit increases in Nox activity, it was proposed that H_2O_2 -mediated vasodilation in the cerebral microcirculation may play a protective role against endogenous vasoactive hormones, thereby preventing cerebral vasospasm and subsequent neuronal damage. In agreement with this postulate, Miller et al. showed that AngII initiated significantly smaller contractions in cerebral arteries than in systemic vessels, which were potentiated by both catalase and apocynin [50]. In addition, Nox2 was identified to be an important regulator of cerebral blood flow *in vivo*, as NADPH-induced cerebral vasodilation and increase in blood flow were significantly attenuated by Nox2 inhibitor gp91ds-tat as well as in gp91^{phox}-null mice [150]. Another physiological contribution of Nox-derived ROS was reportedly flow-induced vasodilation in cerebral arteries [151,152]. It was reported in cultured primary cells [153], in isolated arteries [152] as well as *in vivo* [151] that Nox activity in the endothelium is enhanced by shear stress. Moreover, the evidence that flow-mediated ROS production and vasodilation could be inhibited by catalase and DPI points to the contribution of Nox-generated H_2O_2 to flow-dependent responses in cerebral circulation [151]. Further mechanistic studies of Nox and ROS-mediated cerebral vascular relaxation revealed mediators both in smooth muscle and endothelium. Sobey et al. reported that dilation of rat cerebral arterioles in response to H_2O_2 is mediated by activation of large conductance calcium-activated potassium (BK_{Ca}) channels in vascular smooth muscle cells. This was proved by selective blockers of these potassium channels (TEA and iberiotoxin) largely inhibiting a putative H_2O_2 -induced vasorelaxation [51]. In agreement with this finding, Cheranov et al. utilized patch clamping to demonstrate that TNF α activates Nox in cerebral smooth muscle cells, leading to production of H_2O_2 activating BK_{Ca} channel currents and resulting in a reduction in global intracellular Ca²⁺ concentration and vasodilation [154]. In cerebral arterial endothelial cells, Nox-derived ROS were found to propagate the lipid peroxidation metabolite 4-HNE, which further initiated Ca²⁺ influx through TRPA1 channels, activation of intermediate-conductance Ca²⁺ channel sparklets and vasodilatation [155].

In addition to facilitating vasodilation under physiological conditions, Nox can mediate cerebral vasoconstriction in response to increases in intraluminal pressure, i.e. myogenic tone. Gebremedhin et al. made key discoveries that increased intraluminal pressure markedly promotes superoxide and H_2O_2 production in isolated cerebral arteries, which are inhibited by Nox2 inhibitor gp91ds-tat. Elevated ROS levels in smooth muscle cells further evoke redox-sensitive signaling mechanisms involving crosstalk between inactivation of the dual phosphatase PTEN and activation of the PI3K/Akt pathway. This, in turn, inhibits BK_{Ca} channels, leading to depolarization and pressure-induced vasoconstriction of cerebral arteries [156]. The seemingly paradoxical involvement of Nox in both dilation and constriction of cerebral arteries in fact appears to highlight the significance of Nox in the fine tuning of cerebral vascular tone and local blood flow. Further, this dichotomy might be attributed to different isoforms of Nox, Nox localization combined with the degree of ROS

generated and distinct downstream signaling pathways. Elucidation of these mechanisms will likely require rigorous and directed application of (a) isoform-specific Nox inhibitors; and/or (b) ROS scavengers.

3.3. Cerebral microvascular Nox in disease

3.3.1. Endothelial dysfunction—It is well characterized that ROS impairs cerebral vascular function primarily via inhibition of the endothelium-dependent NO signaling pathway, resulting in dysregulated cerebral microcirculation [157,158]. For example, acetylcholine-induced CBF increase is attenuated by AngII treatment. This process is reversed by apocynin and Nox2 inhibitor, and absent in Nox2 deficient mice, all supportive of the key involvement of Nox2 in AngII-elicited inhibition of NO signaling of the cerebral microvasculature [59]. Indeed, the interaction between superoxide anion and NO not only reduces NO bioavailability, hence diminishing its vasodilatory capability, but also gives rise to peroxynitrite (ONOO⁻) that leads to damage of DNA, lipids and protein, and more profound vascular pathologies. Several groups have shown that peroxynitrite is implicated in cerebrovascular dysfunction, including impaired NO-dependent vasodilation [159] and augmented vasoconstrictor response during post-ischemic reperfusion [160]. Consistent with the effects on endothelial cells, high concentration of ROS in cerebral arterial smooth muscle also promotes cell contraction and vasoconstriction. Amberg et al. reported that exogenous ROS increases L-type calcium channel activity in isolated myocytes via activation of PKC [161]. As signaling pathways in endothelium and smooth muscle act in concert to facilitate Nox-mediated cerebral vascular dysfunction, Noxs can serve as important targets for therapeutic development against cerebral microvascular diseases.

3.3.2. BBB disruption—Several studies have shown that ROS and Noxs are involved in blood brain barrier (BBB) disruption from various causes. It was reported that Nox inhibition and genetic deletion of Nox2 attenuates stroke-induced BBB disruption and lesions *in vivo* as well as increases in permeability of brain capillary endothelial cells *in vitro* [162]. It was also found that apocynin pre-treatment prevents traumatic brain injury-induced ROS production and BBB disruption, thus decreasing neuronal death [21]. Amyloid β (A β), whose accumulation in brain capillaries is observed in 40% of the Alzheimer's disease cases, was found to significantly increase ROS generation and capillary tight junction alterations, both reversed by administration of DPI and an ROS scavenger lipoic acid [163]. Furthermore, increase in BBB permeability *in vitro*, as determined by FITC-dextran leakage through a monolayer of cultured human brain microvascular endothelial cells (HBMECs), is observed following lipopolysaccharide (LPS) treatment [164]. Concomitantly, LPS potentiated p67^{phox} and p47^{phox} expression, elevated Nox activity and reduced tight junction protein expression. These adverse effects on BBB integrity were all attenuated by the Nox inhibitor apocynin, indicating that Nox, as the primary source of ROS activated by LPS, is a significant regulator of BBB function [164]. Moreover, ROS production and BBB damage were also reported in oxidized low-density lipoprotein-stimulated cerebral arteries [165] and in AngII-treated stroke-prone SHR rats [166]. In both models, Nox inhibition effectively diminished ROS generation and BBB disruption, demonstrating that Nox-dependent oxidative stress represents a common instigator for cerebral microcirculatory BBB dysfunction. Thus, targeting NADPH oxidases may be an

effective therapeutic strategy for preventing BBB disruption or restoring its integrity. Although mechanisms underlying Nox-mediated BBB disruption have not been fully elucidated, implications of phosphatidyl inositol 3-kinase, c-Jun N-terminal kinase, Rho kinase in ROS-impaired permeability have been reported [162]. Interestingly, involvement of Rho kinase appears to be both upstream and downstream of Nox and ROS signaling in the cerebral microvasculature, as Rho kinase inhibitor not only prevents ROS-induced BBB damage, but also attenuates oxygen-glucose deprivation-related increases in Nox2 expression and ROS production, restores intercellular tight junctions and preserves BBB integrity [162,167].

3.3.3. Inward remodeling—Another key cerebral vascular abnormality resulting from oxidative stress is alteration in arteriolar structure. It is well acknowledged that hypertension is associated with inward eutrophic remodeling, which is characterized by decreased outer and luminal diameter and increased media/lumen ratio [168]. This is often observed in resistance-size arteries in the brain [169] as well as peripheral vascular beds [170]. As blood flow is proportional to the fourth power of the vessel's internal radius, slight changes in lumen diameter can profoundly affect tissue perfusion. Therefore, inward remodeling can exert profound effects on cerebral blood flow. Oxidative stress caused by hypertension is a key mediator of microvascular remodeling in various vascular beds such as mesenteric and subcutaneous arterioles [2]. However, ROS-initiated structural changes in the cerebral vasculature and its potential contribution to vascular function or cerebral blood flow are poorly understood. Chan et al. utilized Nox2 knockout mice to show that superoxide anion generated from the Nox2 isoform oxidase plays a key role in AngII-induced cerebral arteriolar inward remodeling [171]. Nevertheless, the intracellular mechanisms underlying this process require further scrutiny.

3.3.4. Neurovascular uncoupling—Cerebral parenchymal arterioles branch off from upstream arteries on the surface of the brain (pial arteries) and penetrate into the cerebral cortex, where they are encased by astrocytes and neurons. Increased neuronal activity signals through astrocytes, and subsequently leads to arteriolar dilation and an increase in local cerebral blood flow satisfying enhanced glucose and oxygen demand; a process termed neurovascular coupling or functional hyperemia [172]. Neurovascular coupling is disrupted in a variety of pathological conditions, including hypertension, Alzheimer's disease and ischemic stroke [173], all of which are also associated with elevated levels of arteriolar ROS [20,174,175]. Compelling evidence has shown that Nox2-derived superoxide in the arterioles contributes heavily to impairment of functional hyperemia induced by AngII [20]. AngII evokes significant ROS production in cerebral microvessels and attenuates cerebral blood flow increase from whisker stimulation. These effects were largely inhibited by pegylated-superoxide dismutase (SOD), Tiron, apocynin, and were prevented in mice treated with Nox2 inhibitor gp91ds-tat or in Nox2 deficient mice [20,176]. Aging also represents an important factor for compromised neurovascular coupling, which is believed to cause further age-decline in cortical function and vascular cognitive impairment [177,178]. Further, Toth et al. showed that the cerebral cortex in aged mice had significantly higher mRNA expression of Nox1, Nox2 and Nox4 subunits compared to young mice, also associated with increased level of oxidative stress [179]. Moreover, a whisker stimulation-evoked CBF

increase was remarkably lower in aged mice. Both of these effects were inhibited by apocynin, supporting the notion that Nox plays a central role in impairment of neurovascular function and dysregulation of cerebral microcirculation [179]. However, the signaling pathways controlling ROS-mediated damage in neurovascular coupling remain to be unveiled.

3.3.5. Aging—In fact, in addition to compromised functional hyperemia that is briefly described above, it is well documented that aging is associated with diverse cerebrovascular alterations that include reduction in resting CBF, disrupted microvascular integrity, atrophic structural changes and attenuated responses to endothelium-dependent vasodilators [158,180–182]. All these alterations increase the susceptibility of the cerebral vasculature to functional abnormalities, ischemic injury and vascular cognitive impairment [180]. Numerous studies have established a major contribution of Noxs and oxidative stress in age-related vascular dysfunction of the cerebral circulation. For example, aging is associated with increased ROS production in the cerebral cortex and cerebral arteries [158,183], where mRNA transcript levels of Nox subunits such as Nox2, p47^{phox}, and p67^{phox} are elevated in aged groups [158,184]. Further, mice lacking Nox2 do not exhibit cerebrovascular oxidative stress, and are protected from disruption of endothelium-dependent relaxation and impairment of functional hyperemia [158,183]. ROS scavenger Tempol, non-selective Nox inhibitor apocynin and selective Nox2 inhibitor gp91ds-tat all ameliorate aging-induced ROS production and improve vascular function in aged animals [158,183–185]. Recently, the correlation between aging and Alzheimer's disease was rigorously interrogated, as they, together with hypertension, are purportedly major determinants of cognitive impairment. Aging serves as a powerful risk factor for the cognitive decline caused by hypertension and Alzheimer's Disease. Therefore, it is not surprising that the contribution of hypertension, Alzheimer's Disease and aging to cerebrovascular dysfunction and cognitive decline are intertwined and highly interactive. Burgeoning evidence reveals that elevated vascular oxidative stress from Nox appears to be a common thread associated with these conditions [186,187]. In support of this notion, Park et al. utilized aged Tg2576 mice, a mouse line that overexpresses a mutant form of amyloid precursor protein (APP), and is linked to the pathogenesis of Alzheimer's disease and progressive cognitive deficits. They reported that aging and amyloid deposition, both alone and together, cause cerebrovascular dysfunction including attenuated functional hyperemia and reduced endothelium-dependent dilator responses. Deleting Nox2 not only rescued these abnormal vascular functions in full, but also diminished oxidative stress in aged Tg2576 mice. Similarly, application of Nox2 inhibitor gp91ds-tat largely improved neurovascular coupling and vasodilatory responses in these mice [174]. These reports suggest that Nox2 is a central player in cerebral microcirculatory dysfunction from age-related pathological conditions. In support of these findings, a recent study from Han et al. showed that both apocynin and Tempol reduced ROS levels and improved responses to vasoactive stimuli in aged Tg2576 mice [188]. They also ameliorated vascular smooth muscle-dependent hypercontractile responses observed in aged Tg2576 mice. The authors further noted that the anti-ROS therapy decreased cerebral amyloid angiopathy and micro-hemorrhage that commonly occur in Alzheimer's Disease [188]. As all these studies point to the key contribution of the Nox, particularly Nox2, to aging-elicited cerebrovascular dysfunction, Nox2 inhibition holds great promise as a

therapeutic strategy [52,189–191] for restoring vascular health in cerebral microcirculation, and hopefully delaying the onset of cognitive impairment.

4. Nox inhibitors as therapeutics for microvascular diseases

4.1. Peptidic inhibitors

4.1.1. Nox2ds-tat—Since its initial design and characterization [192], Nox2ds-tat (previously gp91ds-tat) has been one of the most widely used isoform-specific inhibitors of the Nox2 isozyme. It selectively blocks the interaction between Nox2 and p47^{phox} by binding to the latter and preventing p47^{phox} translocation to the plasma membrane. We have previously shown that Nox2ds-tat is a potent inhibitor of the canonical Nox2 oxidase with an IC₅₀ of 0.74 μ M, and a selective inhibitor with no effects on Nox1, Nox4 or xanthine oxidase activity [52]. Moreover, the evidence that Nox2ds-tat inhibits neither the canonical Nox1-NoxO1 binding nor the hybrid Nox1-p47^{phox} interaction further demonstrates its specificity on the Nox2 isozyme [52]. Since its development, Nox2ds-tat has proven its value not only in delineating the involvement of Nox2-derived ROS in cardiovascular biology as well as in myriad other organ systems physiology, but also in attenuating Nox2/ROS-mediated cell and tissue damage *in vitro* and *in vivo* [193–206]. In resistance arteries from hamster gracilis muscle, the physiological contribution of Nox2 to elevated intraluminal pressure-induced ROS generation, Ca²⁺ sensitivity and myogenic vasoconstriction was revealed by application of Nox2ds-tat [29]. Moreover, Nox2 was identified as an important source of superoxide anion in the cerebral arteries, as Nox2ds-tat, but not control scrambled peptide, significantly attenuated superoxide anion production in response to increased intraluminal pressure [156]. In addition, Nox2ds-tat was shown to attenuate myogenic tone in afferent arterioles from spontaneous hypertensive rats, indicating that Nox2-derived ROS play a critical role in potentiating the myogenic response in hypertension [81]. Furthermore, since Nox2ds-tat inhibits bradykinin-induced dilation in coronary arteries as well as superoxide anion and H₂O₂ generation in coronary artery endothelial cells, Nox2 is proposed as an essential enzymatic source of H₂O₂ that mediates agonist-induced vasodilation in the microcirculation [62]. On the other hand, the therapeutic potential of Nox2ds-tat in the microcirculatory system has also been well described. For example, in a type2 diabetes model, Nox2ds-tat not only was able to attenuate EGF receptor activation-enhanced Nox activity in vascular cells, but also improved EGF-initiated endothelial dysfunction in mesenteric resistance arteries including the rescue of eNOS and Akt signaling [19]. Studies on the functional benefits of Nox2ds-tat are particularly revealing in the cerebral microcirculation. Several reports have shown that Nox2ds-tat mitigates the impairment of neurovascular coupling/functional hyperemia in mice evoked by various pathological stimuli [20,176,183]. These include cerebral flow changes in response to beta amyloid protein [176] and are consistent with the role of Nox2 in behavioral changes resulting from overexpression of beta amyloid [174]. Nevertheless, a commonly raised disadvantage of the inhibitor is its expected poor oral bioavailability. Testing of emergent technologies for better stabilization (i.e. peptide stapling [207,208] and nanoparticle formulations [209–218]) will likely be required to improve the druggability of Nox2ds-tat.

4.1.2. NoxA1ds—NoxA1ds was developed to mimic a putative “activation domain” of Nox1 activator subunit NoxA1 which is homologous to a reported p67^{phox} “activation domain”, thus blocking the Nox1-NoxA1 binding and isozyme activation [219]. Previous work characterizing NoxA1ds in our laboratory has corroborated the selectivity of this peptidic inhibitor, which potently inhibited Nox1-derived superoxide anion production in a reconstituted Nox1 cell-free system with an IC₅₀ of 19 nM, and exhibited no inhibitory effects on Nox2-, Nox4-, Nox5-, or xanthine oxidase-derived ROS production [219]. NoxA1ds was shown to inhibit hypoxia-induced ROS generation and endothelial cell migration in human pulmonary arterial endothelial cells [219]. It also reduced uniaxial cyclic stretch-induced Nox1-dependent superoxide anion generation and synthetic phenotypic switch in rat aortic smooth muscle cells [84]. Although no evidence for beneficial effects of NoxA1ds in the microcirculation is yet available, administration of the inhibitor is expected to attenuate microvascular changes potentially mediated by Nox1, such as AngII-elicited vascular hypertrophy [17] and ischemia-reperfusion injury-induced potentiation of vasocontractile response in mesenteric resistance arteries [73].

4.2. Small molecule inhibitors

4.2.1. Diphenylene iodonium (DPI) and apocynin—Both compounds are traditionally and widely utilized as broad spectrum Nox inhibitors to investigate the roles of Noxs in various cardiovascular pathologies. Nevertheless, as both agents generate sizable off-target effects, studies using these inhibitors alone should be subjected to more scrutinized review. Specifically, apart from its inhibitory effects on Noxs, DPI is also demonstrated to be an irreversible and nonselective inhibitor of a wide array of flavin-dependent enzymes, such as nitric oxide synthase and xanthine oxidase [220]. Apocynin was initially believed to inhibit Noxs by specifically preventing p47^{phox} translocation to the plasma membrane [220]. This has been called into question by a reported direct scavenging activity of apocynin of non-radical oxidant species such as HOCl and H₂O₂ [221,222] as well as its ability to suppress expression of various Nox catalytic subunits and/or inhibit Nox isozymes that do not require p47^{phox} [222,223]. These nonspecific actions of DPI and apocynin largely diminish their value in dissecting the roles of Noxs in redox signaling pathways. Rather, one or more isoform selective inhibitors or siRNAs ought to be employed in addition to DPI and apocynin to clearly validate the participation of Nox. On a positive note, these broad spectrum inhibitors notwithstanding have exhibited protective effects against microvascular diseases. For example, apocynin and DPI suppressed ROS formation and restored endothelial function in aortas as well as cerebral arterioles from aged rats [144,158]. Moreover, apocynin conferred protection against neurovascular uncoupling arising with age [179]. It was also reported that traumatic brain injury-initiated ROS elevation, BBB disruption and neuronal death can all be prevented by apocynin [21]. However, whether these beneficial effects are entirely attributable to Nox blockade warrants further investigation.

4.2.2. VAS2870 and VAS3947—VAS2870 and VAS3947 are triazolopyrimidine derivatives. They have been characterized as promising Nox inhibitors based on the observation that they reduce Nox-derived ROS generation in several cell lines, including primary vascular endothelial and smooth muscle cells, with no antioxidant properties or

inhibition of xanthine oxidase or eNOS activity [224–227]. VAS2870 is considered a *pan*-Nox inhibitor as it suppresses the activity of Nox1, Nox2, Nox4 and Nox5 [224,228]. Its preclinical effectiveness was evaluated by several groups who reported that VAS2870 improved microvascular function in a variety of vascular beds. For instance, experimental ischemia/reperfusion in middle cerebral arteries significantly upregulated Nox2 and Nox4, potentiated ROS production, and caused BBB leakage and brain edema in hyperglycemic rats, which were all markedly blunted by VAS2870 treatment [229]. Moreover, the expression of several key modulators in BBB integrity was decreased following ischemia/reperfusion, and was partially restored by VAS2870 [229]. VAS2870 was shown to reverse attenuated endothelium-dependent vasodilation of mesenteric arteries from type 2 diabetic mice and insulin-resistant rats, as well as arteries treated with amylin, a peptide strongly associated with insulin resistance [230,231]. However, off-target effects of VAS2870 on the cellular thiol redox status, i.e. thiol alkylation, were also reported [232]. In other words, when assessing the roles of Nox in physiological and pathological settings, findings obtained with VAS2870 should be interpreted with further caution. As with all inhibitors, the combination of various pharmacological as well as genetic tools will likely provide more definitive conclusions.

4.2.3. GKT13690 and GKT137831—Both compounds are pyrazolopyridine derivatives. They are Nox inhibitors effective for dual Nox1/Nox4, but not for Nox2, suppression [233]. Moreover, they do not show detectable inhibitory actions against other ROS-generating and redox-sensitive enzymes [233]. Due to high oral bioavailability, ease of synthesis and a reportedly good safety profile, GKT137831 has been proposed as a new therapy for diabetic kidney disease and has undergone phase 2 clinical trials [234]. In the microvasculature, GKT136901 was reported to ameliorate impaired vasodilatation of mesenteric arterioles from obese (db/db) mice displaying enhanced Nox1 expression [235], suggesting that this dual Nox1 and Nox4 inhibitor could be useful therapeutically for treating microvascular dysfunction in a range of cardiovascular diseases.

4.2.4. ML171—ML171 was the first small molecule reported to be an isoform-selective Nox1 inhibitor [236]. ML171 does exhibit inhibitory effects on Nox2, Nox3, Nox4 and xanthine oxidase but with an approximate 20-fold lower potency than that for Nox1. Incidentally, it exerts no apparent inhibitory effects on mitochondrial ROS generation [236]. The relative isoform selectivity renders this compound a useful tool (when a relatively low concentration of ML171 is employed) to elucidate the contribution of Nox1 to redox-signaling. For example, a recent study on the effects of chronic restraint stress on vascular function showed that ML171 (0.5 μ M) reduced AngII-initiated vasoconstriction in rat carotid arteries in the presence and absence of endothelium, implying that Nox1-derived ROS positively influence AngII-mediated contraction in carotid arterial smooth muscle cells [237]. Another study comparing endothelial function of coronary arteries from male and female pigs reported that 100 μ M ML171 enhanced bradykinin-induced endothelium-dependent relaxation in male rather than female porcine coronary arteries [238]. Nevertheless, it is not clear whether Nox1 alone played a role in suppressing vasodilation, since 100 μ M of ML171 is high enough to abolish the activity of other Nox isozymes as well as xanthine oxidase [236].

4.2.5. Ebselen—Ebselen was initially characterized as a glutathione peroxidase mimetic [239] and a scavenger of peroxynitrite [240]. Later, inhibitory actions of ebselen congener JM-77b on Noxs were reported with relatively higher selectivity for Nox2 ($IC_{50}=0.4\ \mu M$) compared to Nox1 ($IC_{50}=6.3\ \mu M$), Nox4 (no detectable inhibition) and Nox5 ($IC_{50}=17\ \mu M$) [241]. Its ability to inhibit Nox2 notwithstanding, the antioxidant properties of ebselen, *per se*, were favorably utilized in different disease models to improve vascular reactivity. In a study on hypoxia/reoxygenation-initiated brain damage, ebselen ameliorated endothelial dysfunction in rat posterior cerebral arteries following hypoxia/reoxygenation insults [242]. Additionally, administering ebselen for 7 days significantly reduced vasospasm in right internal carotid arteries and middle cerebral arteries after subarachnoid hemorrhage in primates [243]. It was also reported to restore voltage-gated potassium (K_v) channel expression and activity in coronary arteriolar smooth muscle cells where channels were suppressed in diabetic rats. Consequently, ebselen potentiated K_v channel-dependent vasodilation in small coronary arteries [244]. However, whether or not any of these protective functions is associated with Nox inhibition is not entirely clear.

4.2.6. Tetrahydroquinolines—In light of the profound implication of Nox2 in myriad macro- and microvascular pathologies, and perception of poor clinical applicability of peptidic Nox2ds-tat, development of small molecule Nox2-specific inhibitors has long been a quest for use as both an investigative tool and a therapeutic strategy. Using high throughput screening and rational design, our laboratory identified bridged tetrahydroquinolines as specific Nox2 inhibitors [191]. Among a group of structurally related molecules, compounds **11 g** [(±)-(1S,4R,9S)-5-bromo-3,3-dimethyl-9-(2-methylallyl)-10-pentyl-1,2,3,4-tetrahydro-1,3-(epiminomethono) naphthalene] and **11 h** [(±)-(1S,4R,9S)-5-bromo-3,3-dimethyl-9-(2-methylallyl)-10-(thiophen-2-ylmethyl)-1,2,3,4-tetrahydro-1,4-(epinomethano)naphthalene] displayed isoform-selective inhibition on Nox2 in intact COS-Nox2 cells with IC_{50} of 20 μM and 32 μM , respectively. Importantly, 11 g and 11 h had no inhibitory actions on ROS production by Nox1-, Nox4-, Nox5-expressing systems or xanthine oxidase, and showed no free radical scavenging activity [191]. Unpublished work from our group suggests that these inhibitors likely interfere with Nox2-p47^{phox} binding via blockade of the p22^{phox}-p47^{phox} interface. Functionally, 11 g and 11 h significantly inhibit TNF α -induced inflammatory activation in aortic endothelial cells and endothelial dysfunction in mouse aorta (unpublished). Although they have not been evaluated on the microcirculation, these inhibitors hold great promise in improving Nox2-mediated microvascular complications.

Other Nox inhibitors include S17834 [245] and AEBSF [246], both of which decrease superoxide formation by Nox oxidases but also display multiple off-target effects [220]. Fulvene-5 [247] was also shown to reduce Nox2 and Nox4 activity, however, further interrogation on its specificity for Nox isozymes, mechanism of action, and effectiveness on the cardiovascular system is not reported. In summary, even though important strides in discovering Nox inhibitors have been made during the past decade, selective and clinically applicable Nox inhibitors are scarce and require further assessment of their mechanisms of action, pharmacokinetic properties and *in vivo* efficacy. Inhibitors specifically for Nox3, Nox4, Nox5, DUOX1/2 are not yet available, which may be a consequence of a poor

understanding of interactions of this subset of Noxs with intracellular subunits. Indeed, further research developing more Nox modulators is warranted as they will not only advance the field of Nox research but also provide novel hit compounds that might be manipulated in clinical settings.

5. Conclusions and future perspectives

The dynamic network of the microcirculatory system is of vital importance to the modulation of blood flow and perfusion pressure locally and systemically. Impaired microvascular function often leads to vascular remodeling, tissue ischemia and organ damage. Structural and functional abnormalities of the microcirculation, as observed in various cardiovascular diseases, are strongly associated with an imbalance of the redox environment. As the likely major source of ROS in the vascular system, Noxs are important signaling molecules that participate physiologically in the regulation of microvascular tone. All the more crucial, excessive production of ROS (resulting from increased Nox expression or activity) plays a central role in triggering microvascular remodeling, endothelial dysfunction, increased BBB permeability and impaired neurovascular coupling in both the systemic and cerebral microcirculations. These manifestations are all hallmarks of cardiovascular disease including those evident in hypertension, diabetes, stroke and aging. The central involvement of Nox-derived ROS signaling in microvascular function and pathology is depicted in Fig. 1. Despite their importance, understanding of Nox and ROS as cellular signaling agents at the level of the microcirculation is far from sufficient. One important limiting factor lies in the paucity of a broad array of specific tools to delineate the individual roles of Nox isoforms. With the development of selective Nox1 and 2 inhibitors, the roles of the Nox1 and Nox2 isozymes are poised to be the best characterized in the vasculature in coming years. More work is needed, however, to develop novel, isoform-selective inhibitors (peptidic or small molecule) with optimal pharmacokinetic and pharmacodynamic profiles for each of the Nox isozymes. This is particularly critical for Nox5, both owing to its absence of expression in rats and mice, rendering it impossible to utilize conventional gene deletion models, and because Nox5 is abundantly expressed in human vascular cells and implicated in a growing number of human cardiovascular pathologies. Moreover, considerably more work is required to gain mechanistic insights into the regulation of Nox-mediated redox signaling pathways in the microcirculatory system. In particular, questions related to what specific Nox isoforms are involved, how signaling cascades are differentially regulated, how Noxs integrate the complex network of signaling and govern cellular and vascular function and whether these experimental findings can be translated into human diseases, require immediate attention. It is also noteworthy that Nox/ROS-implicated redox pathways remain far less interrogated in cerebral vs. other microvascular cells, which may be a consequence of limited tissue mass and low yield of signaling proteins. While most research focuses on the functional aspects of Noxs in arteriolar activity, more mechanistic studies of the Nox in cultured primary microvascular cells is critical. Finally, a better appreciation of the complexity of these signaling pathways will lead to the development of novel and effective therapeutic strategies aimed at preventing and treating microvascular pathologies in humans.

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Fig. 1.

Major Nox-mediated redox-sensitive signaling pathways involved in microvascular physiology and pathophysiology. Schematic diagram illustrating known Nox and ROS-mediated signaling pathways in cerebral and systemic microvascular endothelium and smooth muscle. Arrows denote positive activation of downstream targets. Blunt-ended arrows indicate inhibitory effects on targets. Dashed arrows denote pathways that are active in other vascular systems but have not been reported in the microcirculation. In endothelial cells, Noxs can be activated by hormones, cytokines, growth factors and oscillatory shear stress [248,249], which stimulate ROS-dependent pathways that eventually lead to increased angiogenesis, inflammation, BBB permeability and impaired neurovascular coupling. Interaction between superoxide anion and NO yields peroxynitrite, which reduces NO bioavailability and causes endothelial dysfunction. In smooth muscle cells of cerebral and systemic arterioles, Noxs can be stimulated by mechanical stretch as well as vasoactive agents, e.g. AngII. Nox-derived ROS are centrally involved in inhibiting potassium channels, activating protein kinases and increasing Ca²⁺ sensitivity of the contractile apparatus. These effects are physiologically important for myogenic tone regulation, but may also lead to augmented vasoconstriction and diminished vasorelaxation. Microvascular remodeling is also dependent on Nox-mediated ROS production through putative stimulation of MMPs. Abbreviations: O₂^{•-}: superoxide anion; H₂O₂: hydrogen peroxide; SOD: superoxide dismutase; eNOS: endothelial nitric oxide synthase; NO: nitric oxide; ONOO⁻: peroxynitrite; p38: p38 mitogen-activated protein kinases; JNK: c-Jun N-terminal kinases; ERK: extracellular signal-regulated kinases; HIF1 α : hypoxia-inducible factor 1- α ; AMPK: 5' AMP-activated protein kinases; NF κ B: nuclear factor- κ B; GPCRs: G protein-coupled receptors; TRP channels: transient receptor potential channels; PKC: protein kinase C; ROCK: rho-associated protein kinase; ROS: reactive oxygen species; BK: big-conductance Ca²⁺-activated K⁺ channels; K_v: voltage-gated K⁺ channels; PKG: cGMP-dependent protein kinase; GTP: guanosine triphosphate; cGMP: cyclic guanosine monophosphate; sGC: soluble guanylyl cyclase; MMP: matrix metalloproteinases.

Table 1

Nox family of proteins in the microcirculation.

<i>Nox isozyme*</i>	<i>Canonical subunits/regulators</i>	<i>ROS Generation</i>	<i>Tissue expression in the microvasculature^a</i>
Nox1	p22 ^{phox} , NOXO1, NOXA1, Rac1/2	Superoxide anion.	Mouse: mesenteric arteries [58], cerebral microvessels [59] Rat: mesenteric arteries [17,60], basilar arterial endothelium [61]; Human: coronary arteriolar endothelium [62], cerebral arteriolar smooth muscle [63]
Nox2	p22 ^{phox} , p47 ^{phox} , p67 ^{phox} , p40 ^{phox} , Rac1/2	Inducible; Superoxide anion.	Mouse: mesenteric arteries [19,58], coronary arteries [64], afferent arterioles [65]; cerebral microvessels [59] Rat: mesenteric arteries [66,67], basilar arterial endothelium [61]; Human: coronary arteriolar endothelium [62], renal arterioles [68], subcutaneous arteriolar smooth muscle [43], cerebral arteriolar smooth muscle [63].
Nox4	p22 ^{phox} , Poldip2 [49]	Constitutively active; Capacity for primary production of H ₂ O ₂ [69].	Mouse: mesenteric arteries [19,58], cerebral microvessels [59]; Rat: mesenteric arteries [60], basilar arterial endothelium [61]; Human: renal arterioles [68], coronary arteriolar endothelium [62], subcutaneous arteriolar smooth muscle [43], cerebral arteriolar smooth muscle [63]
Nox5	No subunits identified to date	Superoxide anion; Independent of p22 ^{phox} ; regulated by Ca ²⁺ .	Human: coronary arteriolar endothelium and smooth muscle [57,70]

^a As primarily identified by its core catalytic subunit comprising cytochrome b558.