Review

Vascular Nadph Oxidases and Atherothrombotic Stroke

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Review

Vascular NADPH Oxidases and Atherothrombotic Stroke

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Abstract: Oxidative stress constitutes a main molecular mechanism underlying cardiovascular diseases (CVDs). This pathological mechanism can be triggered by NADPH oxidases (NOXs), which produce reactive oxygen species (ROS). In fact, the different NOXs have been associated with myocardial infarction, atherothrombosis, and stroke. More specifically, we will focus on the implications of NOXs in atherothrombotic stroke. Each NOX member participates in a different way in the several stages of this disease: endothelial dysfunction, immune cell infiltration, foam cell genesis, vascular smooth muscle cells (VSMC) proliferation, and atherosclerotic plaque formation. Additionally, some NOXs are involved in plaque instability, thrombosis, ischemic stroke, and ischemia-reperfusion injury (IRI). Interestingly, the effects of NOXs in this pathology depend on the specific homologue, the cell type in which they are activated, and the stage of the disease. In this review we summarize the most up-to-date information about the implications of vascular NOXs in each of these processes. Finally, we highlight some limitations and future perspectives of the study of NOXs in CVDs.

Keywords: oxidative stress; NADPH oxidases; NOX; atherosclerosis; thrombosis; stroke; vessel wall; atherothrombosis

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1. Introduction

Atherothrombotic stroke is a cardiovascular disease (CVD) with a high global incidence. It occurs when a thrombus forms after the rupture of an atherosclerotic plaque and it travels across the bloodstream, finally obstructing blood flow in the brain parenchyma [1]. This disease progresses through several phases, beginning with the development of an atherosclerotic plaque. Initially, endothelial cells are damaged by humoral stimuli, leading to inflammation and the transfer of low-density lipoproteins (LDL) into the vessel intima [2]. Reactive oxygen species (ROS) produced from different cell types oxidize these LDLs (oxLDLs). Immune cells attracted by the damaged endothelium infiltrate and accumulate oxLDLs, becoming foam cells [3]. These cells accumulate and secrete growth factors, causing the invasion of the subendothelial space by vascular smooth muscle cells (VSMC). VSMC proliferate and switch their phenotype (sometimes becoming foam cells), contributing to plaque development. This plaque can either occlude the artery, or rupture, leading to thrombus formation, which could potentially cause an ischemic stroke [1].

One of the most relevant molecular mechanism underlying atherothrombotic stroke is oxidative stress, characterized by an imbalance between ROS levels and antioxidant defenses in the cells. In this context, ROS overreact, modifying lipids, proteins, and DNA, and cause cell damage, which may trigger different pathologies [4]. ROS are mainly produced by the mitochondrial electron chain, the NADPH oxidase family (NOX), nitric oxide synthases, xanthine oxidase, and cytochrome P450 [5]. Interestingly, the NOX family is the primary ROS source in the vasculature and its overactivation is a key factor in several CVDs, such as atherothrombotic stroke [6].

2. NADPH Oxidases in the Vessel Wall

NOXs constitute a family of enzymes that produce ROS as their main catalytic product. This family is composed of seven different members: five homologues (NOX1-NOX5) and two dual oxidases (DUOX1, DUOX2). These members differ in their regulatory subunits, their expression across different cell types, and their intracellular localization [7]. Specifically, NOX1, NOX2, NOX4 and NOX5 are expressed in the vascular wall and monocytes/macrophages, while DUOXs present lower levels [8], and NOX3 is restricted to the inner ear [9]. In this section we summarize the distribution of the main NOX members in endothelial cells, VSMC and monocytes/macrophages (Figure 1).

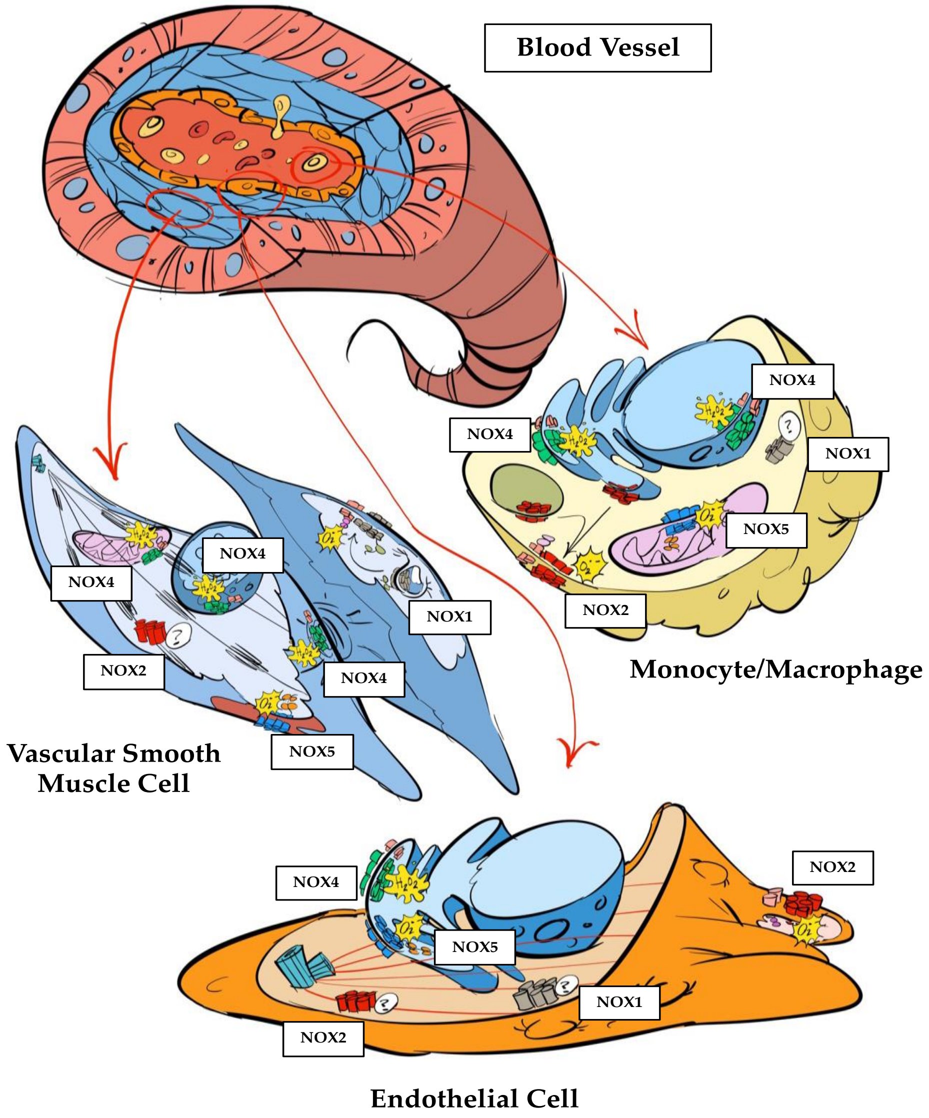

Figure 1. Intracellular distribution of NOX homologues in endothelial cells, VSMC and monocytes/macrophages. Endothelial Cells. NOX1 is expressed, but its intracellular localization remains unknown. NOX2 locates in membrane protrusions and in association with the cytoskeleton. NOX4 and NOX5 locate in the endoplasmic reticulum. **Vascular Smooth Muscle Cells.** NOX1 locates inside clatrin-coated areas of the membrane when inactive, and translocases to the membrane upon activation. NOX2 is expressed, but its intracellular location remains unknown. NOX4 locates inside the mitochondria, at the nucleus and at focal adhesions. NOX5 locates at cholesterol-rich areas of the plasma membrane. **Monocytes/Macrophages.** NOX1 is expressed, but its intracellular localization remains unknown. NOX2 locates at intracellular membranes and translocases to the plasma

membrane upon activation. NOX4 locates at the endoplasmic reticulum and the nucleus. NOX5 locates at mitochondria.

NOX1 was described in 2000 in mammalian cell lines [10]. Its activation requires of regulatory subunits (p47phox, NOXA1), and its main product is $O_2^{\bullet-}$. NOX1 is expressed by endothelial cells [11] and VSMC [12] in the vascular wall, and by monocytes [13]. Intracellularly, NOX1 has been detected in the nucleus, the plasma membrane, endosomes, and peroxisomes [14]. In VSMC, NOX1 is expressed in caveolin-coated areas of the plasma membrane [15]. Little is known about its localization in endothelial cells and monocytes. NOX1 upregulation has been associated with CVDs such as atherosclerosis, hypertension, and diabetes [16–18].

NOX2 (gp91phox or phagocytic NOX) was the first described member and it produces $O_2^{\bullet-}$. It is a highly glycosylated protein, and its activation depends on phosphorylation and the recruitment of p22phox, p47phox and p67phox subunits. NOX2 is expressed in endothelial cells, VSMC and monocytes in different subcellular localizations [14]. For instance, in macrophages, NOX2 is located at intracellular membranes at baseline, translocating to the plasma membrane upon activation [19]. In endothelial cells, NOX2 associates with the actin cytoskeleton and cellular protrusions [20]. By contrast, NOX2 localization in VSMC remains unknown. NOX2 has been associated with diabetes, myocardial infarction, and thrombosis [21,22].

NOX4 (initially named “Renox”) was firstly described in the kidney [23]. NOX4 only requires p22phox subunit to produce ROS and appears to be active under physiological conditions [24]. The primary effect of this oxidase is mediated by H_2O_2. NOX4 is expressed in endothelial cells [25], VSMC [16], and monocytes [26]. In endothelial cells and macrophages, it locates in the endoplasmic reticulum [20,26]; and in VSMCs, in focal adhesions, mitochondria, and the nucleus [15,27,28]. There is some debate about the role of NOX4 in CVDs, as it has been described to display both protective and damaging roles [29].

NOX5 was the last discovered member of the family. It is highly expressed in the testis, spleen, and lymph nodes [30], but it is absent in the rodent genome, which complicates its study in the experimental rodent models. Interestingly, NOX5 is the only member regulated by Ca^{++} levels. In the human vasculature, NOX5 is present at all vascular segments [31]: endothelial cells [32], VSMC [33] and monocytes [34]. It locates in the nucleus, endoplasmic reticulum, and plasma membrane [35]. In VSMC it is abundant in cholesterol-rich areas of the plasma membrane and translocates to rafts upon activation [36]. In dendritic cell-derived monocytes, NOX5 is expressed on the outer membrane of the mitochondria [37]. Recently, NOX5 has been found to interact with the actin cytoskeleton [38]. NOX5 is implicated in several CVDs such as diabetes, atherosclerosis and myocardial infarction [39].

3. NADPH Oxidases in Atherosclerosis

3.1. NADPH Oxidases and Endothelial Dysfunction

NOX members are activated by humoral factors associated with CVDs, leading to endothelial dysfunction. For example, Ang II stimulates NOX2 [40,41], NOX4 [42], and NOX5 [43,44] activity/expression in human endothelial cells. ET-1 activates NOX2 and NOX5-derived ROS in porcine and human endothelial cells respectively [43,45], contributing to endothelial dysfunction. Moreover, oxLDLs induce NOX2 activity and endothelial dysfunction in human primary coronary artery endothelial cells [46]. The role of NOX4 in endothelial dysfunction in response to LDL/oxLDL stimulation is debated; some studies describe a pathological role in human umbilical vein endothelial cells (HUVEC) [47,48], while others suggest a protective role in LDL-receptor *knock-out* mice [49]. Finally, lysophosphatidylcholine activates NOX5 increasing Ca^{++} levels in human aortic endothelial cells (HAEC) [50].

In addition, NOXs are involved in endothelial cell apoptosis. For instance, NOX1 regulates apoptosis in human sinusoidal endothelial cells [11]. The localization of NOX2 and NOX4 appears closely linked to apoptosis in HUVEC. Specifically, caspase 3 activity associates with NOX2- and NOX4-derived ROS production in response to homocysteine [51]. Finally, our group previously showed that NOX5 induces apoptosis in HAECs and immortalized human brain microvascular endothelial cells (hBMECs) [52,53].

These proapoptotic effects of NOXs create a proinflammatory environment by releasing cytokines from endothelial cells. For instance, NOX2 in endothelial cells mediates the *in vivo* production of granulocyte-monocyte colony stimulating factor (GM-CSF), likely through toll-like receptor (TLR) stimulation [54]. Likewise, NOX4 potentiates the secretion of IL-8 and monocyte chemoattractant protein 1 (MCP-1) upon TLR-4 activation in human endothelial cells, as well as induces IL-6 production in response to palmitate [55–57]. Moreover, endothelial NOX5 in diabetic Akita mice increased inflammation via MCP-1 and TLR4 [58]. Tobacco smoke extract induced CX(3)CL1 production via NOX5 activation in HUVEC, increasing immune cell adhesion [59]. Prostaglandins (PGs) are also implicated in this process. In HUVEC, NOX4 inhibits the protective effects of PGI_2, related with different vasculopathies [60]. Finally, in immortalized HAEC (teloHAEC), NOX5 increases PGE_2 production by COX-2 activation [44]. All the mentioned studies are summarized in Figure 2.

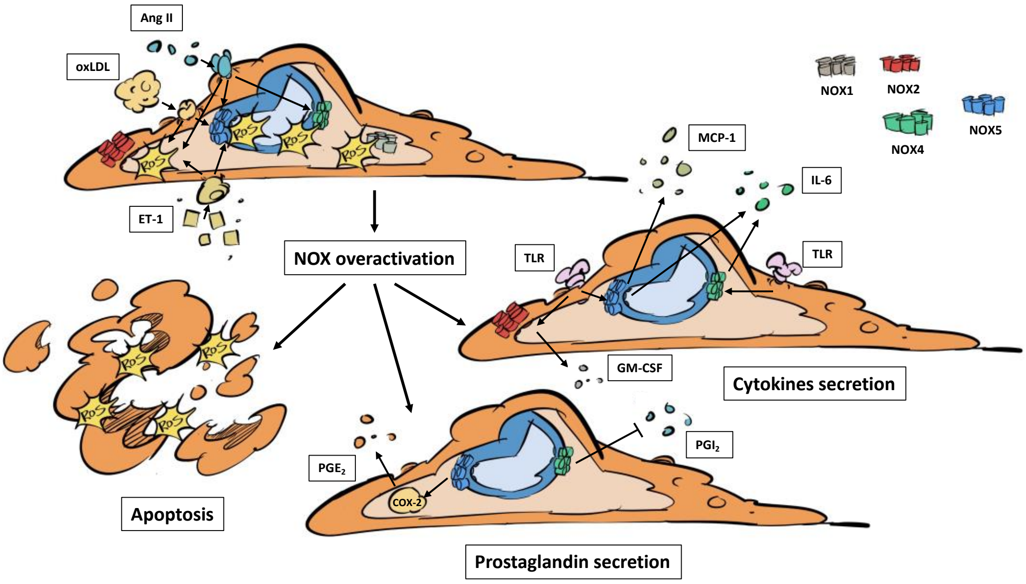

Figure 2. NOX overactivation leads to endothelial cell dysfunction and inflammation. Humoral factors related with atherosclerosis (Ang II, ET-1, oxLDL) increase NOX activity. The overactivation of every NOX leads to apoptosis. The activation of NOX2, NOX4 and NOX5 by TLRs ends in cytokines secretion. NOX4 and NOX5 alter PGs signaling, inflammatory mediators that participate in atherosclerosis. Ang II: angiotensin II. ET-1: endothelin 1. GM-CSF: granulocyte-monocyte colony stimulating factor. MCP-1: monocyte chemoattractant protein 1. oxLDL: oxidized LDL. PGE_2: prostaglandin E_2. PGI_2: prostaglandin I_2. TLR: toll-like receptor.

3.2. NADPH Oxidases, Immune Cell Infiltration and Foam Cells

In this proinflammatory context, endothelial cells upregulate adhesion molecules to facilitate immune cell adhesion and infiltration. NOX activity and expression closely correlates with adhesion molecule expression. NOX2 overexpression in murine endothelial cells increases leukocyte adhesion and VCAM-1 expression. In mice with cardiac hypertrophy produced by Ang II infusion, NOX2 enhances immune cell infiltration into the heart [61]. Similarly, salusin-β (a prohypertensive peptide) increases monocytic adhesion in VSMC through NOX2 [62]. By contrast, the overexpression of NOX2 in monocytes does not promote adhesion to endothelial cells [63]. Again, NOX4 overexpression in endothelial cells exhibits a protective role, reducing the recruitment of immune cells caused by Ang II *in vivo* [64]. However, NOX4 also promotes the expression of ICAM-1 and VCAM-1 in response to TNF-α in HUVEC [65]. Finally, NOX5 increases VCAM-1 and ICAM-1 expression, and mononuclear cell infiltration in HUVEC [66]. This pathway could be induced by oxLDLs [50]. In a myocardial infarction model, mice overexpressing NOX5 in endothelial cells upregulate cardiac VCAM-1 levels [67].

Changes in the extracellular matrix (ECM) can facilitate immune cell infiltration into the subendothelial space of the vessel wall. Interestingly, p22phox regulates vascular wall elastic fiber composition *in vivo*, correlating with the MMP-12/TIMP-1 ratio [68]. More interactions between NOX enzymes and matrix metalloproteinases (MMPs) are known. For instance, Ang II-induced NOX1 activation promotes VSMC migration and proliferation via MMP-9 [69]. NOX2 activation by Ang II induces vascular remodeling in VSMC and adventitial fibroblasts [70,71]. Besides, NOX2 is implicated in the development of the atherosclerotic plaque *in vivo*, which seems to be related with a reduction in the MMP-9 activity [72]. Finally, the Ang II-NOX4 axis influences the effects of adventitial fibroblasts in the vascular ECM [73]. The relationship between NOX5 and MMP expression/activity in the vascular context remains unclear.

NOXs also play a crucial role in foam cell formation by mediating lipid oxidation. NOX1 may play a dual role in this process. On the one hand, NOX1 activation by TLRs increases ROS production and foam cell generation [74,75]. On the other hand, NOX1 participates in atherosclerotic plaque propagation by mediating LDL pinocytosis [76]. NOX2 has been implicated in macrophage apoptosis during efferocytosis, contributing to increased inflammation and immune cell infiltration into the plaque [77]. Furthermore, NOX2 promotes lipid accumulation in VSMC, another source of foam cells [62]. There is no literature suggesting a direct relationship between NOX4 or NOX5 and foam cells, although NOX5 has been associated with VSMC migration in hypertensive patients [78]. All these studies are summarized in Figure 3.

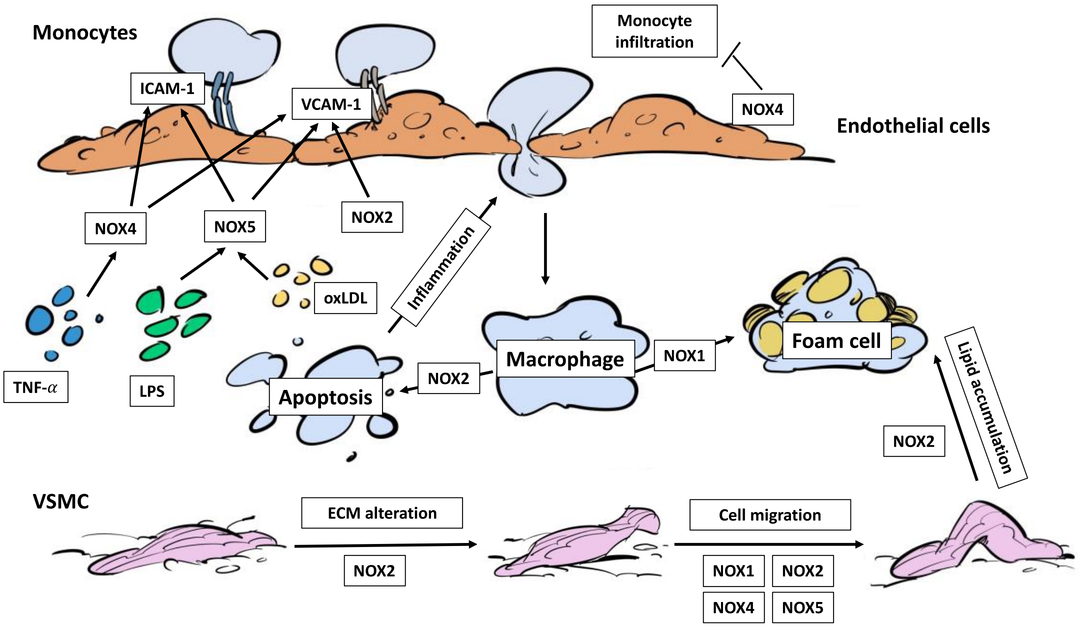

Figure 3. The different NOX homologues play crucial roles in the immune cell infiltration into the vascular wall and the genesis of foam cells. NOX2, NOX4 and NOX5 increase ICAM-1 and VCAM-1 expression in endothelial cells, promoting monocyte adhesion. By contrast, NOX4 inhibits the infiltration of monocytes. NOX2 promotes the apoptosis of macrophages which aggravates the immune cell infiltration by inflammatory signals. NOX1 mediates the transformation of macrophages towards foam cells. NOX2 activation leads to alterations in ECM composition, cell migration and lipid accumulation in VSMC. NOX1, NOX4 and NOX5 also participate in VSMC migration. ECM: extracellular matrix. ICAM-1: intracellular adhesion molecule 1. LPS: lipopolysaccharide. TNF-α: tumoral necrosis factor α. VCAM-1: vascular cell adhesion molecule 1.

3.3. NADPH Oxidases, Plaque Development, Plaque Rupture, and Thrombosis

Foam cells stimulate neighboring VSMC, promoting their migration and proliferation within the atherosclerotic plaque, finally increasing its size. NOX1A mediates VSMC migration, proliferation,

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and differentiation into macrophage-like cells in ApoE$^{-/-}$ mice [79]. These effects of NOX1 on VSMC have been widely described *in vitro* and *in vivo* [80–82]. Interestingly, NOX2 activity follows NOX1 in VSMC, promoting cell migration through a second peak of ROS [70]. Although the effect of NOX2 on VSMC migration was already described [62], this study highlights a temporal relationship between two NOXs in a specific cell type.

NOX2 expressed in endothelial cells contributes to vascular remodeling by increasing VSMC proliferation, indicating a paracrine effect derived from NOX activity [83]. NOX2 in VSMC induces IL-17A production, potentially promoting a phenotypic switch [84]. The precise role of NOX4 in VSMC migration and differentiation remains unclear, some studies suggest an anti-synthetic and anti-proliferative role [85], while others propose a pro-migratory one [86]. Although limited information exists on NOX5 and VSMC, great progress has been made recently. VSMC extracted from hypertensive patients present greater migratory capacity mediated by NOX5 activation [87]. Regarding vascular calcification, NOX5 promotes the switch of VSMC to a synthetic phenotype that secretes Ca^{++}-loaded vesicles [88]. This effect of NOX5 in VSMC-mediated vascular calcification may appear in humans in response to smoking [33].

Several *in vivo* models of atherosclerosis served to evaluate the effects of NOXs on plaque development. NOX1 produces pro-atherosclerotic effects in diabetic mice, as its deletion decreases chemokines secretion, immune infiltration, and profibrotic markers expression [89]. Interestingly, ET-1 production from endothelial cells is linked to NOX1 pro-atherosclerotic effects *in vivo* [90]. Dietary supplementation with blackberry prevented NOX1 pathological effects *in vivo* [91]. NOX2 plays a pro-atherosclerotic role in early stages of the disease by reducing NO availability [92]. NOX2 inhibition by gp91ds-tat causes the regression of the atherosclerotic plaques in ApoE$^{-/-}$ mice [72]. NOX4 deletion in ApoE$^{-/-}$ mice accelerates atherosclerosis in response to partial carotid artery ligation under a high-fat diet, suggesting a protective role [93]. Conversely, in LDLr$^{-/-}$ mice, NOX4 exhibits a pathological profile in response to a high-fat diet, increasing plaque burden [49]. NOX4 deletion in VSMC prevents plaque development in response to Ang II [94]. In coronary artery disease patients, NOX5 appears overexpressed in atherosclerotic plaques, localizing in endothelial cells in early lesions and in VSMC in advanced lesions [31]. Furthermore, NOX5 colocalizes with immune cells in plaques from patients with atherosclerosis [95]. Regarding experimental atherosclerosis, endothelial overexpression of human NOX5 in *knock-in* mice does not affect plaque development [96]. However, NOX5 deletion in rabbits aggravates atherosclerosis in response to high-fat diet [97]. Therefore, it is unclear if NOX5 promotes pathology or acts in response to vascular damage.

NOXs can also modulate the stability of atherosclerotic plaques. NOX1 activation in VSMC promotes the development of unstable plaques [98]. In a model combining carotid branch ligation, renal artery constriction and high fat diet in ApoE$^{-/-}$ mice, NOX2 inhibition favors the production of more stable plaques [99]. NOX4 expression in patients with carotid artery stenosis directly correlates with plaque stability and inversely correlates with caspase-3 activity, suggesting a protective role [100]. The role of NOX5 in plaque stability remains unknown.

The instability of atherosclerotic plaques leads to rupture and thrombosis. In this stage of the disease, NOXs play a crucial role. Pharmacological inhibition or genetic depletion of NOX1 reduces collagen-dependent thrombosis in a $FeCl_3$-induced carotid occlusion model [101]. However, studies where NOX1 is specifically depleted from platelets suggest that NOX1 is involved in thrombin-induced thrombosis. By contrast, NOX2 seems to be involved in collagen-induced thrombosis [102]. On the contrary, another group describes that NOX2 is dispensable for arterial thrombosis in large vessels [103]. Finally, in a carotid occlusion model comparing a triple *knock-out* mouse (NOX1$^{-/-}$, NOX2$^{-/-}$ and NOX4$^{-/-}$) to NOX-specific *knock-out* mice, it was concluded that NOX4 does not play a relevant role in pulmonary thromboembolism and *ex vivo* platelet aggregation, while NOX1 and NOX2 act as prothrombotic enzymes [104]. The role that NOX5 plays in thrombosis remains unclear. As it can be observed, there is still some controversy regarding the role of NOXs in thrombosis. All the described studies are summarized in Figure 4.

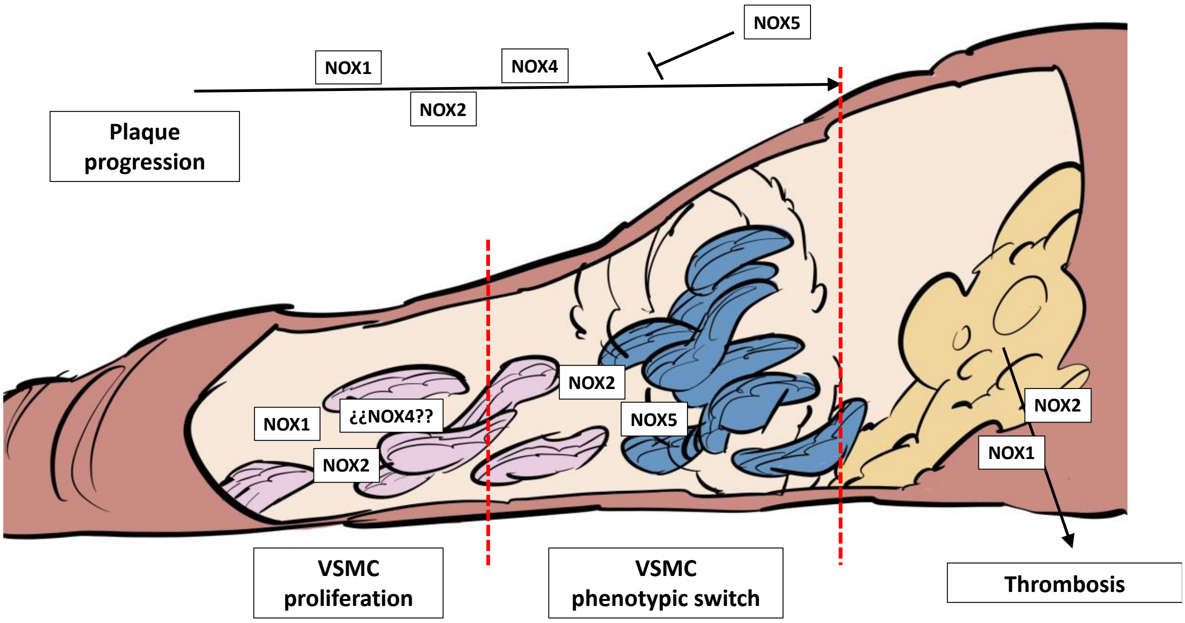

Figure 4. The NOX homologues play crucial roles in atherosclerotic plaque growth and thrombosis. NOX1 and NOX2 increase VSMC proliferation, while the role of NOX4 in this step is controversial. NOX2 and NOX5 promote VSMC switch toward a synthetic phenotype and Ca^{++} accumulation. NOX1, NOX2 and NOX4 promote plaque progression *in vivo*, while NOX5 inhibits this process in rabbits. NOX1 and NOX2 are prothrombotic, while NOX4 has no effect in this process.

4. NADPH Oxidases in Thrombosis and Stroke

4.1. NADPH Oxidases, BBB Disruption and Stroke

Once the thrombus occludes a cerebral artery, an ischemic stroke occurs. In this context, NOX1 seems to alter BBB permeability. Oxygen/glucose deprivation increases the ROS production by NOX1 in hBMEC, reducing the expression of adherent proteins and promoting permeability [105]. GKKT136901, a NOX1/4 inhibitor, prevents the increased permeability of hBMEC caused by methamphetamine, restoring ZO-1 and VE-cadherin expression [106]. In a model of traumatic brain injury, NOX1 is upregulated in endothelial cells from the neurovascular unit, overproducing $O_2^{\bullet-}$ and increasing permeability by the dysregulation of the TJs [107]. However, the relevance of NOX1 in middle cerebral artery occlusion (MCAO) models seems to be limited [108,109].

NOX2 presents similar effects on BBB permeability and stroke. At oxygen and glucose deprivation conditions, NOX2-derived ROS promote hBMEC permeability [110]. Likewise, methamphetamine induces NOX2 activation, dysregulating ZO-1, occludin and claudin 5, and increasing BBB permeability in rat microvascular endothelial cells *in vivo* [111]. In an ischemic stroke model, NOX2 expression increased in the brain twelve hours, one day and two days after the reperfusion. Interestingly, NOX2 *knock-out* mice subjected to ischemic stroke present lower infarct volume and edema, and improved neurological outcomes compared to their control littermates [112]. Nevertheless, other studies suggest a limited role for NOX2 in these models [108].

Little information is available about the role of endothelial NOX4 in BBB permeability. NOX4 expression increases after stroke in human patients and experimental models. NOX4 *knock-out* mice subjected to MCAO are prevented from BBB leakage and neuronal apoptosis. Similar effects are observed when using VAS2870, a specific NOX4 inhibitor [108]. Finally, endothelial NOX4 *knock-out* mice exhibit smaller infarct sizes after MCAO [113].

Finally, the role of NOX5 in BBB disruption and stroke remains poorly understood. Our group showed that in aged endothelial NOX5 *knock-in* mice, ZO-1 and occludin expression decreased, which was accompanied by memory loss [114].

4.2. NADPH Oxidases and Immune Infiltration in the Brain

After the onset of ischemia, immune cells infiltrate the brain in coordinate temporal waves. Initially, infiltrative immune cells present a proinflammatory phenotype, turning into anti-inflammatory resolving cells at later stages of the disease [115]. Scarce information is available about NOXs effects at this point. Immortalized hBMEC exposed to the organic pollutant PCB153 increased ICAM-1 and VCAM-1 expression through a NOX-dependent mechanism. Interestingly, apocynin and diphenyleneiodonium chloride, two flavoprotein inhibitors, prevented these effects [116]. NOX2 is related with the developmental process of microglia in the cerebral cortex of mice. More specifically, NOX2 promotes the infiltration of macrophages in the developing tissue, indicating a role in peripheral immune cells infiltration into the brain [117]. In a model of chronic restrain stress, several NOX subunits and proinflammatory markers appear upregulated in the cerebrovascular endothelium, indicating a relationship between NOXs and cerebral inflammation during adult life [118].

In other CVDs the findings are similar. In a subarachnoid hemorrhage model, splenectomy reduces the infiltration of inflammatory cells and NOX2 expression in cardiac and cerebral tissues [119]. NOX activity seems to mediate ATP-derived NETosis after MCAO, an activity produced by infiltrating neutrophils [120]. Besides, NOX2 negatively regulates TIPE-2, a protein that inhibits the infiltration of peripheral immune cells [121]. Although scarce, the works carried out to date suggest that NOXs might play a role in the infiltration of immune cells during ischemic stroke.

4.3. NADPH Oxidases and Ischemia-Reperfusion Injury

NOXs have been deeply studied in the ischemia-reperfusion injury (IRI), a damage associated with an excessive production/accumulation of ROS after the recanalization of the infarcted area. Some authors suggest that NOX1, NOX2 and NOX4 play a relevant role in this process, since their expression increase after reperfusion in a nylon-induced MCAO model. In this study, PI3K$\gamma^{-/-}$ mice inhibit the upregulation of NOXs, which resulted in reduced neutrophil infiltration, MMP-9 expression and brain damage [122]. Nonetheless, in a similar model of IRI in rats, NOX2 and NOX4 expression, but not NOX1, were upregulated at protein levels in the ischemic tissue [123].

Surprisingly, ROS produced during IRI may develop a dual role. In a mouse model of MCAO and reperfusion, NOX-inhibition by apocynin after damage displayed different effects depending on the timing. After the resolution of stroke, apocynin reduces inflammation and promotes angiogenesis. However, one week and two weeks after the event, it increases inflammation and reduces angiogenesis [124].

NOX1 mediates ROS production after oxygen/glucose deprivation and reoxygenation in murine brain endothelial cells [105]. In male rats subjected to MCAO followed by reperfusion, NOX1 expression increases in neurons of the peri-infarcted region. Interestingly, NOX1 inhibition increases newborn cells survival in this region, improving functional recovery [125].

In a similar model, NOX2 *knock-out* mice present increased revascularization of the infarcted area after three days [126]. Then, miR-652 reduced NOX2 expression, ROS production and tissue injury one day after reperfusion in the brain of rats subjected to MCAO [127]. NOD2 seems to activate NOX2 during IRI *in vivo*, increasing the secretion of proinflammatory mediators in the initial days after reperfusion [128]. Moreover, NOX4 targeting by miR-454 reduced ROS production in neuron-like SH-SY5Y cells, exhibiting a protective effect. Likewise, this miRNA reduced infarct size, edema, and cell death in the brain of rats one day after reperfusion [129]. Besides, NOX4 has been associated with TLR4 activation one day after IRI. TLR4 inhibition reduced NOX4 expression, oxidative stress, and neuronal apoptosis *in vivo* [130]. Interestingly, several drugs that exert neuroprotective effects against IRI in rodents seem to act by downregulating NOX4 [131–133]. Additionally, apocynin and NADPH treatment protected the brain tissue from inflammation and injury in an IRI model, a protection mediated by NOX2 and NOX4 downregulation [134]. NOX2 and NOX4 overexpression seem to be acute responders to IRI, as their levels increase three hours after reperfusion, returning to baseline after one day [135].

There is little information available about NOX5 implications in IRI. Nonetheless, Casas et al., recently described a key role for this oxidase. NOX5 produces ROS in the first hour after reoxygenation, while NOX4 acts later with a peak of activity after four hours. Interestingly, hypoxia increased NOX5 expression in hBMEC. Besides, in an immune and endothelial cell *knock-in* model for NOX5, BBB leakage and infarct volume were increased, and the neurological outcomes worsened after reperfusion [136].

5. Conclusions

The atherothrombotic stroke is strongly influenced by NOX activity in each stage. NOX members promote endothelial dysfunction, immune cell infiltration, foam cell genesis, atherosclerotic plaque development, thrombosis, BBB disruption, cerebral inflammation and IRI. Although NOXs have been widely studied, there is still a great lack of information from different approaches:

(i) More cell type-specific *knock-out/knock-in in vivo* models would help to improve the current knowledge.
(ii) More integrative studies that deep in the interconnection between different NOXs or their paracrine effects should be performed.
(iii) There is an urgent need to develop isoform-specific NOX inhibitors and study these enzymes as potential therapeutical targets in CVDs.

To sum up, although great efforts have been made to study NOXs in atherothrombotic stroke, there is still a lot to discover.

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Conflicts of Interest: The authors declare no conflicts of interest.

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