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Review

NADPH oxidase-derived reactive oxygen species in the regulation of endothelial phenotype

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Abstract:
Endothelial dysfunction comprising impairment of endothelium-dependent vasodilator function and increased endothelial activation contributes to the pathophysiology of cardiovascular diseases such as atherosclerosis, diabetic vasculopathy, heart failure and hypertension. The changes in endothelial phenotype in these conditions occur in response to diverse stimuli including inflammatory cytokines, activation of renin-angiotensin-aldosterone system, hyperlipidaemia, hyperglycemia, ischemia-reperfusion and mechanical forces. An increased production of reactive oxygen species (ROS), such as superoxide and H_2O_2, is involved in the genesis of these alterations in endothelial phenotype. The NADPH oxidases, Nox2 and Nox4, are major sources of ROS in endothelial cells and are implicated both in vasodilator dysfunction and in the modulation of redox-sensitive signalling pathways that influence endothelial cytoskeletal organisation, adhesion molecule expression, permeability, growth, migration and other functions. NADPH oxidases appear to be especially important in redox signalling in that they are specifically activated by diverse agonists and regulate the activation of downstream protein kinases, transcription factors and other biological molecules. This review provides an overview of NADPH oxidase structure and regulation in endothelial cells and their role in pathophysiology, focussing particularly on endothelial activation.

Key words:
endothelial dysfunction, activation, ROS, NADPH oxidase, redox signalling

Abbreviations: ACE – angiotensin converting enzyme, BMP – bone morphogenetic protein, eNOS – endothelial nitric oxide synthase, ERK – extracellular regulated kinase, H_4B – tetrahydrobiopterin, HMG-CoA – 3-hydroxy-3-methylglutaryl-coenzyme A, JNK – c-Jun N-terminal kinase, LPS – lipopolysaccharide, MCP-1 – monocyte chemoattractant protein-1, MKP-1 – mitogen activated protein kinase phosphatase-1, MMP – matrix metalloproteinase, NFκB – nuclear factor κB, NO – nitric oxide, oxLDL – oxidised low density lipoprotein, ROS – reactive oxygen species, TLR – toll-like receptor, TNF-α – tumor necrosis factor-alpha, TRAF – TNF receptor associated factor, WAVE-1 – WASP-family verprolin homologous protein, VCAM-1 – vascular cell adhesion molecule-1, VEGF – vascular endothelial growth factor, VSMC – vascular smooth muscle cell

Introduction

The endothelium, a single layer of cells that lines the lumen of all blood vessels and the heart, is well known to play an important physiological role in vascular homeostasis. Endothelial cells not only form a semi-permeable barrier between blood and the vessel wall but also have crucial (patho)physiological functions, including: (a) the modulation of vascular tone; (b) maintenance of vascular integrity and blood fluidity *via* regulation of thrombosis, fibrinolysis and platelet aggregation; (c) regulation of inflammation

and immune responses *via* alterations in vessel wall-inflammatory cell interactions and permeability; (d) contribution to the maintenance of a quiescent, differentiated vascular smooth muscle cell (VSMC) phenotype; and (e) involvement in neovascularisation. These functions are mediated through the synthesis and release of various paracrine factors (eg, nitric oxide [NO], prostacyclin, endothelin, endothelium-derived hyperpolarising factors, chemotactic molecules and growth factors) and the expression of surface molecules such as angiotensin converting enzyme (ACE), tissue plasminogen activator, and cell adhesion molecules [10].

Abnormalities of endothelial function are implicated in the pathophysiology of several cardiovascular conditions including atherosclerosis, diabetic vasculopathy, hypercholesterolaemia, heart failure and hypertension. An increase in oxidative stress, denoting an imbalance between reactive oxygen species (ROS) production and antioxidant defence, is now well recognised to play an important role in the genesis of endothelial dysfunction and recent studies implicate NADPH oxidases as major sources of ROS involved in this abnormality [10, 39]. In this article, we review the role of NADPH oxidase-derived ROS in endothelial cells, focussing particularly on endothelial activation.

ROS and endothelial dysfunction

Dysfunction of the endothelium encompasses both abnormalities of endothelial-dependent vasodilator regulation and endothelial activation, a term that is used to describe specific and complex changes in endothelial phenotype which involve an increase in endothelial-leukocyte interactions (among other effects) and are central to pathophysiological inflammatory responses [10, 31]. Endothelial activation is also central to the initiation of atherosclerosis. These changes in endothelial phenotype occur in response to diverse stimuli including inflammatory cytokines, activation of the renin-angiotensin-aldosterone system, mechanical stimuli such as shear stress, ischemia-reperfusion, hyperlipidemia and hyperglycaemia. The presence of impaired endothelium-dependent vasodilatation is an independent predictor of adverse mortality and morbidity outcomes in many clinical settings [9, 34].

A large body of evidence implicates increased ROS in the genesis of both endothelial vasodilator dysfunction and endothelial activation. A major mechanism underlying ROS-dependent impairment of endothelium-dependent vasorelaxation is superoxide-mediated inactivation of vasodilator NO, a reaction that also generates peroxynitrite. Interestingly, oxidative stress leading to endothelial vasodilator dysfunction is also a predictor of adverse outcome in patients with coronary artery disease [28]. In addition to the interaction with NO, ROS (notably H_2O_2) have important direct effects through the modulation of diverse redox-sensitive signalling pathways in endothelial cells which influence gene and protein expression and impact on many different functions [19, 33, 39]. These include endothelial cell growth, proliferation, migration, survival, cytoskeletal reorganisation, cell shape, adhesion molecule expression, permeability and the secretion of inflammatory cytokines.

There are several potential sources of superoxide in endothelial cells, including mitochondria, cytochrome P450-based enzymes, uncoupled eNOS, xanthine oxidase and NADPH oxidases. Among these sources, it is notable that the NADPH oxidases are the only source whose primary function is ROS generation and that they appear to be especially well suited for involvement in redox signalling [12, 37]. In particular, NADPH oxidases are specifically activated by many of the stimuli that are known to cause endothelial dysfunction and activation. Recent studies confirm an important role for NADPH oxidases as major sources of ROS involved in endothelial dysfunction, activation and redox signalling [12, 37]. These enzymes have also been found to be important in signal transduction pathways involved in angiogenesis and neovascularisation [56]. Another important attribute of the NADPH oxidases is their potential to augment ROS generation by other enzymes. For example, NADPH oxidases can cause uncoupling of NOS secondary to oxidative degradation of the NOS cofactor tetrahydrobiopterin (H_4B), thereby leading to superoxide rather than NO generation [3]. In addition, xanthine dehydrogenase may be converted to superoxide-generating xanthine oxidase through oxidation by NADPH oxidase-derived ROS [47].

NADPH oxidase structure

The NADPH oxidase complex was first described in neutrophils where it is involved in non-specific host defence against microbes ingested during phagocytosis, through the generation of large quantities of su-

peroxide and protons [37]. The phagocytic oxidase is composed of two membrane-associated subunits, $p22^{phox}$ and $gp91^{phox}$, which form a flavocytochrome (cyt b558). The process of electron transfer from NADPH to molecular oxygen, which results in superoxide formation, is catalysed by the oxidase following its activation through the translocation of several cytosolic regulatory subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac), which associate with cytochrome b558. In the last few years, components of the NADPH oxidase were found to be present in many non-phagocytic cells, including VSMC [24], endothelial cells [5], adventitial and cardiac fibroblasts [48] and cardiomyocytes [7]. Differences between the biochemical activity of the phagocytic and non-phagocytic oxidase led to the identification of a whole family of NADPH oxidases based on distinct homologues of $gp91^{phox}$ [37]. These isoforms of $gp91^{phox}$, named Nox1-5 (for NADPH oxidase), are each encoded by different genes and form the basis of different NADPH oxidases (Fig. 1). Nox2 is the name for $gp91^{phox}$ in this new terminology. The Nox family may be classified into two groups, based on predicted domain structures: (i) Nox1-4 all contain six transmembrane domains and have NADPH and FAD-binding domains at the cytoplasmic C-terminus; (ii) Nox5 has a similar basic structure but with an additional N-terminal calmodulin-like Ca^{2+}-binding domain. In addition, 2 related oxidases, Duox1 and 2, include a further N-terminal extension with a peroxidase-homology domain that is separated from the Ca^{2+}-binding domain by an additional transmembrane segment. The expression of these different Nox isoforms varies according to cell and tissue. Nox2 and Nox4 are co-expressed in endothelial cells, although there are a few reports that Nox1 and Nox5 might also be present in some settings [1, 6, 25, 40]. The expression of Nox4 mRNA is reported to be at least 20-fold greater than that of Nox2 [51] although in homogenates of human arteries and veins Nox2 mRNA expression was higher than that of Nox4 [26]. Interestingly, the activation of the Nox4-containing NADPH oxidase does not require any of the conventional cytosolic subunits required for Nox2 activation [2, 46], suggesting that it may be regulated by different stimuli. Additionally, these isoforms may play distinct roles in endothelial cells through expression in different subcellular locations [12, 58].

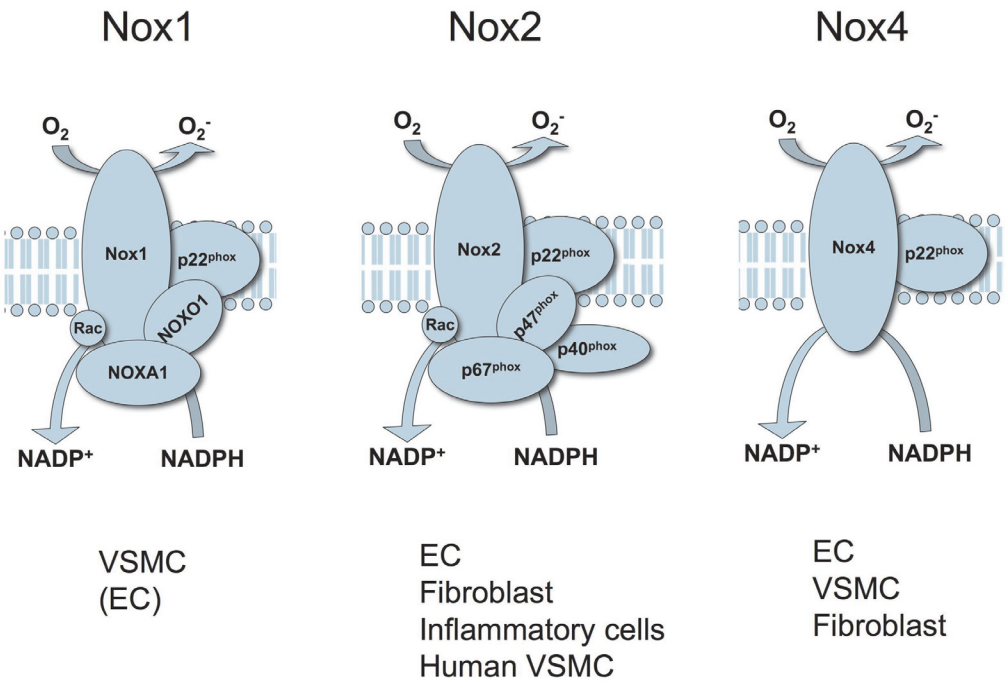

Fig. 1. Schematic diagram of the different NADPH oxidase isoforms and their cellular expression. NOXO1 and NOXA1 are homologues of $p47^{phox}$ and $p67^{phox}$ respectively, which are preferentially required for the activation of Nox1. Nox4 does not appear to require any of these cytosolic regulatory subunits for its activation

Regulation of NADPH oxidase activity

As mentioned above, Nox2 activation requires the translocation of various cytosolic oxidase components [4]. The binding of $p67^{phox}$ to an activation site on Nox2 initiates catalytic activity but interaction of $p47^{phox}$ with $p22^{phox}$ is required to facilitate this process. The binding of activated Rac is also important for full activation of the oxidase. This process is specifically initiated in non-phagocytic cells by diverse stimuli such as angiotensin II, endothelin-1, growth factors (eg, VEGF), and cytokines such as tumor necrosis factor-alpha (TNF-α), mechanical forces and hyperlipidaemia. A key event leading to Nox2 activation is the phosphorylation of $p47^{phox}$, which is essential for its translocation to the membrane cytochrome b558. In endothelial cells, $p47^{phox}$ phosphorylation and oxidase activation have been shown to occur in response to angiotensin II, TNF-α, vascular endothelial growth factor (VEGF), hypoxia-reoxigenation and oscillatory stress [11, 20, 32, 38, 41]. In addition, isoprenylation and activation of the small GTP-binding protein Rac is involved in endothelial oxidase activation in response to altered shear stress, cytokines and ischemia-reperfusion [14, 15, 55]. Isoprenylation of Rac is dependent upon 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity and is inhibited by statins [8, 14, 15, 55], which forms the basis for potential inhibition of NADPH oxidase activity by these agents.

In contrast to Nox2, the Nox4-based oxidase appears to be constitutively active and does not require any of the known regulatory subunits. Stimuli that may activate Nox4 remain to be clearly defined but it has been suggested that it may be activated by lipopolysaccharide (LPS) [49] or insulin [45]. The activity of Nox4 may also be augmented through transcriptional upregulation of mRNA levels. Recently it was suggested that human microvascular endothelial cells also express Nox5, which may be activated by thrombin [6].

NADPH oxidase-dependent redox signalling

An important consideration with regard to Nox-dependent redox signalling is to understand how specificity of signalling could be imparted with such a diffusible mediator such as H_2O_2. Recent studies suggest the concept of compartmentalised ROS production within the cell to explain how signalling specificity may be achieved. Mechanisms that may be involved include the subcellular localisation of each Nox isoform or of pools of the enzyme, the binding of Noxs (or their component subunits) to different scaffold proteins, and compartmentation within endosomal vesicles [12, 19, 38, 42, 57, 58]. It has been reported that Nox2 complexes are present at the plasmalemmal membrane, caveolae and peri-nuclear membranes while Nox4 has been reported to be present in the endoplasmic reticulum and nucleus [20, 30, 36, 40, 41, 61]. Moreover, NADPH oxidase components may interact with a variety of non-oxidase binding proteins to achieve localised superoxide production. It was recently shown that $p47^{phox}$ could interact with TNF- receptor associated factor-4 (TRAF-4), an interaction that was essential for TNF-α-induced activation of extracelluar reglated kinase (ERK)1/2 in human endothelial cells [38]. Another study described protein-protein interactions between $p47^{phox}$ and WASP-family verprolin homologous protein (WAVE-1), an important regulator of the cytoskeleton, which were essential for VEGF-induced c-Jun- N-terminal kinase (JNK) activation [59]. Additionally, the WAVE-1 dependent complex also contained Rac1 and the kinase PAK-1. An interaction between the actin binding scaffolding protein, IQGAP1, and Nox2 and Rac1 was found to be important for VEGF-induced endothelial migration [60]. VEGF-induced IQGAP1-Rac1 binding was suggested to allow spatially localised NADPH oxidase-dependent activation of Akt. Recently, it has been reported that LPS activates Nox4 in endothelial cells *via* a direct interaction between the cytosolic region of the Toll-like receptor 4 (TLR4) and the C-terminal of Nox4 [49].

Involvement of NADPH oxidase in endothelial activation

Important aspects of endothelial activation are the up-regulation of cell surface adhesion molecules such as ICAM-1 (intercellular cell adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1) and E-selectin, chemokines such as monocyte chemoattractant protein-1 (MCP-1), and increases in permeability

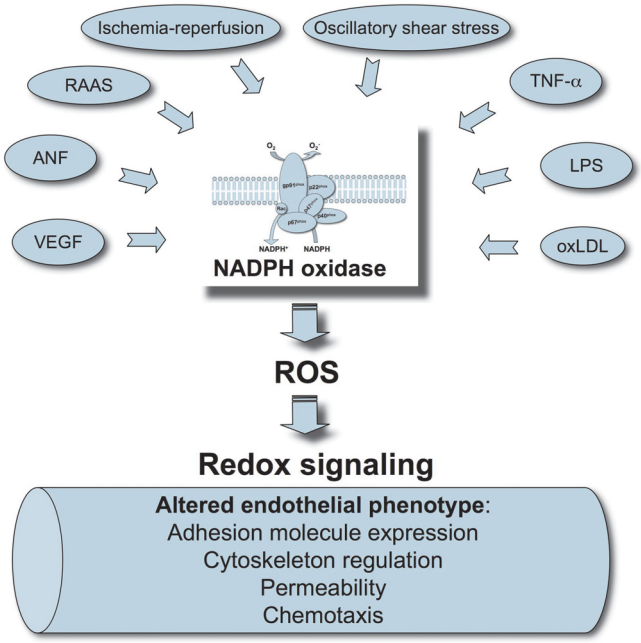

Fig. 2. Schematic diagram showing the role of NADPH oxidase in endothelial cell activation. RAAS = renin-angiotensin-aldosteron system

– all processes known to be influenced by ROS. A significant body of evidence implicates NADPH oxidase activation in these effects (Fig. 2).

TNF-α and other pro-inflammatory cytokines increase the expression of ICAM-1, VCAM-1 and MCP-1 in an nuclear factor-κB (NF-κB) dependent manner *via* the activation of Nox2 [14, 23, 38]. Soluble CD40 ligand (derived mainly from activated platelets) also stimulates ROS production resulting in an upregulation of adhesion molecules in endothelial cells together with an increase in secretion of various chemokines, and matrix metalloproteinase (MMP) expression [29, 44]. LPS induces the expression of surface adhesion molecules through activation of TLR4, which has been shown to involve Nox4-dependent activation of NF-κB [49]. In addition, Park et al. [49] also demonstrated Nox4-dependent increases in monocyte adhesion and transmigration in this study.

Angiotensin II-induced stimulation of the AT_1 receptor is a potent agonist for activation of the Nox2 oxidase in endothelial cells [41]. Several studies have shown that angiotensin II-induced expression of ICAM-1, VCAM-1 and MCP-1 involve NADPH oxidase-derived ROS [17, 43, 50]. Aldosterone also induces adhesion molecule expression *via* mineralocorticoid receptor activation, which has been speculated to involve activation of the Nox4 containing oxidase [27].

Oscillatory shear stress is well known to cause endothelial activation, with this being the likely mechanism underlying the pro-atherogenic effects of disturbed flow in arteries. Increased oscillatory stress increases NADPH oxidase-derived superoxide generation and ICAM-1 expression at least in part secondarily to the effects of bone morphogenic proteins (BMPs) [32]. BMP4 was reported to enhance monocyte adhesion to endothelial cells through the stimulation of NADPH oxidase [52]. In another study, BMP2 also activated endothelial cells through ROS-dependent mechanisms [18].

Interestingly, ANP antagonises TNFα-induced endothelial cell activation, most likely through inhibition of p38MAPK activation via the activation of mitogen activated protein kinase phosphatase-1 (MKP-1) [35]. Recently, Furst et al. [21] reported that this upregulation of MKP-1 involves Nox2-derived ROS. Thus, the Nox2 oxidase could in theory have a dual role by promoting endothelial cell activation as well as inhibiting it, although the precise mechanisms underlying these opposing effects remain unknown.

Hypercholesterolaemia is a key initiating factor for atherosclerosis, at least in part by activating the endo-

thelium and initiating the process of monocyte adhesion and emigration into the vessel wall. It also promotes leukocyte-endothelial cell interactions in postcapillary vessels. Nox2-containing NADPH oxidases, regulated by $p47^{phox}$, have been convincingly shown to be pivotally involved in this process in elegant *in vivo* studies undertaken by the group of Granger [54]. An important stimulus for Nox2 activation in this context may be oxidised LDL (OxLDL) through its binding to lectin-like ox-LDL receptor-1 (LOX-1) [16, 53]. In addition, ROS generated following LOX-1 activation may contribute to cellular proliferation and/or apoptosis [22].

Although we have focussed mainly on endothelial activation in this article, it should be noted that endothelial Nox activation may have several other important effects, notably in the context of angiogenesis and neovascularisation. These processes require multiple changes in endothelial cell phenotype, including cell migration, proliferation and appropriate polarisation to form new vessels. Both tissue hypoxia and VEGF may be important stimuli for Nox2 activation in this setting [13, 56], and an involvement of this isoform in ischemia-induced and VEGF-induced neovascularisation has been confirmed *in vivo* [56, 57].

Conclusions

An increase in endothelial ROS production is involved in the genesis of both endothelial vasodilator dysfunction and endothelial activation, and thereby contributes to the pathophysiology of hypertension, diabetes, atherosclerosis and inflammation. The Nox2 and Nox4 NADPH oxidases are key sources of superoxide involved in these effects and appear to be especially important with regard to redox-sensitive modulation of intracellular signalling pathways. Their involvement in endothelial activation and adhesion molecule expression provides one of the best examples of their role in endothelial pathophysiology. A better understanding of these complex enzymes may allow the development of novel therapies to target abnormal ROS-modulated pathways in endothelial disease.

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